SUPPORTING INFORMATION

Small molecule recognition triggers secondary and tertiary interactions in DNA folding and hammerhead ribozyme catalysis

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S1. Materials and general experimental procedures.

All chemicals were used without further purification from commercial sources, unless otherwise noted. DNAs and labeled RNAs were purchased from Integrated DNA Technologies (IDT) and used without further purification. SYBR® gold was purchased from Thermo Fisher Scientific. DNA stock solutions were serially diluted in deionized water and concentrations were determined by measuring solution absorbance at 260 nm by Thermo Fisher Nanodrop 2000. Sample fluorescence was measured on Thermo Fisher Nanodrop 3300. RNA constructs were prepared by *in vitro* transcription except where noted. The promoter sequence in each DNA template is underlined and does not appear in the final RNA transcripts. 1X PBS buffer was prepared in house with a final concentration of 137 mM NaCl, 2.7 mM KCl and 10 mM PO_4^{3-} at pH 7.4. Tris-Cl buffer was prepared in house with tris(hydroxymethyl)aminomethane, HCl was used to adjust pH to 7.6, final concentration of 1X buffer was 50 mM.

S1.1a) Nucleic acid sequences.

- *DNAs for t2M/t4M binding measurements*

12-T_2-12: 5′-CGC ATA GCT CAG **TT**G ACT CGA TAC GC-3′
3′-GCG TAT CGA GTC **TT**C TGA GCT ATG CG-5′

12-T_4-12: 5′-CGC ATA GCT CAG **TTTT**G ACT CGA TAC GC-3′
3′-GCG TAT CGA GTC **TTTT**C TGA GCT ATG CG-5′

- *U-(2,3) ribozyme:*

Template:
5′-TCA CTG TAA AGA GGT GTT GGT TCT CTT AAT CTT TAA CTT AAA AGG TTA ATG CTA AGT TAG CTT TAC AGT GCG ACA AAA AAA AAA TCT CAA AAA AAA AAG TTT CGA AAA AAT CTC AAA AAA CTC ATC AGG CAC TGC CTA TAG TGA GTC GTA TTA ATT TC-3′
Transcript:
5’-GG CAG UGC CUG AUG AGU UUU UUG AGA UUU UUU CGA AAC UUU UUU UUU UGA GAU UUU UUU UUU GUC GCA CUG UAA AGC UAA CUU AGC AUU AAC CUU UUA AGU UAA AGA UUA AGA GAA CCA ACA CCU CUU UAC AGU GA-3’

- *S-U4n ribozyme:*

Template:
5′-GCC GGG GGT GGG ATT TGA ACC CAC GTA AGG CGG ATC TGC AGT CCG CTG CCT AGC CCC TAG ACT ACC CCG GCT GGT AGA CTG TGA CCC AGT CTC CTG GGT TTC GAA AAC TCA CGA AAA CTC ATC AGA CAG TCT ATA GTG AGT CGT ATT A-3′

Transcript:
5′-G ACU GUC UGA UGA GUU UUC GUG AGU UUU CGA AAC CCA GGA GAC UGG GUC ACA GUC UAC CAG CCG GGG UAG UCU AGG GGC UAG GCA GCG GAC UGC AGA UCC GCC UUA CGU GGG UUC AAA UCC CAC CCC CGG C-3′

- *S-U4n-2bp ribozyme:*

Template:
5′- GCC GGG GGT GGG ATT TGA ACC CAC GTA AGG CGG ATC TGC AGT CCG CTG CCT AGC CCC TAG ACT ACC CCG GCT GGT AGA GCC TGT GAC CCA GTC TCC TGG GTT TCG AAA ACT CAC GAA AAC TCA TCA GAC AGG CTC TAT AGT GAG TCG TAT TA-3′

Transcript:
5′-GAG CCU GUC UGA UGA GUU UUC GUG AGU UUU CGA AAC CCA GGA GAC UGG GUC ACA GGC UCU ACC AGC CGG GGU AGU CUA GGG GCU AGG CAG CGG ACU GCA GAU CCG CCU UAC GUG GGU UCA AAU CCC ACC CCC GGC-3′

- **Binary HHR system enzyme strands**

HH_{min} "Wildtype":
Template:
5′-TGG GTT TCG TCC TCA CGG ACT CAT CAG ACA GTC TAT AGT GAG TCG TAT TA-3′
Transcript (loop II shown in bold):
5′-G ACU GUC UGA UGA GUC C**GU GA**G GAC GAA ACC CA-3′

L-GU3 (GUUU loop)*:*
Template:
5′-TGG GTT TCG TCC AAA CGG ACT CAT CAG ACA GTC TAT AGT GAG TCG TAT TA-3′
Transcript (loop II shown in bold):
5′-G ACU GUC UGA UGA GUC C**GU UU**G GAC GAA ACC CA-3′

L-U4 (4U loop)*:*
Template:
5′-TGG GTT TCG TCC AAA AGG ACT CAT CAG ACA GTC TAT AGT GAG TCG TAT TA-3′
Transcript (loop II shown in bold):
5′-G ACU GUC UGA UGA GUC C**UU UU**G GAC GAA ACC CA-3′

- **Binary HHR system substrate strands** (Scissile bond: *C--A*.)

HH_{min} (wt)*:* 5′ Cy3-UGGGUCA*C--A*GUC**U**CCAAUCC-3′
UU: 5′ Cy3-UGGGUCA*C--A*GUC**UU**CAAUCC-3′
5U: 5′ Cy3-UGGGUCA*C--A*GUC**UUUUU**-3′
Substrate RNAs were used as purchased from IDT.

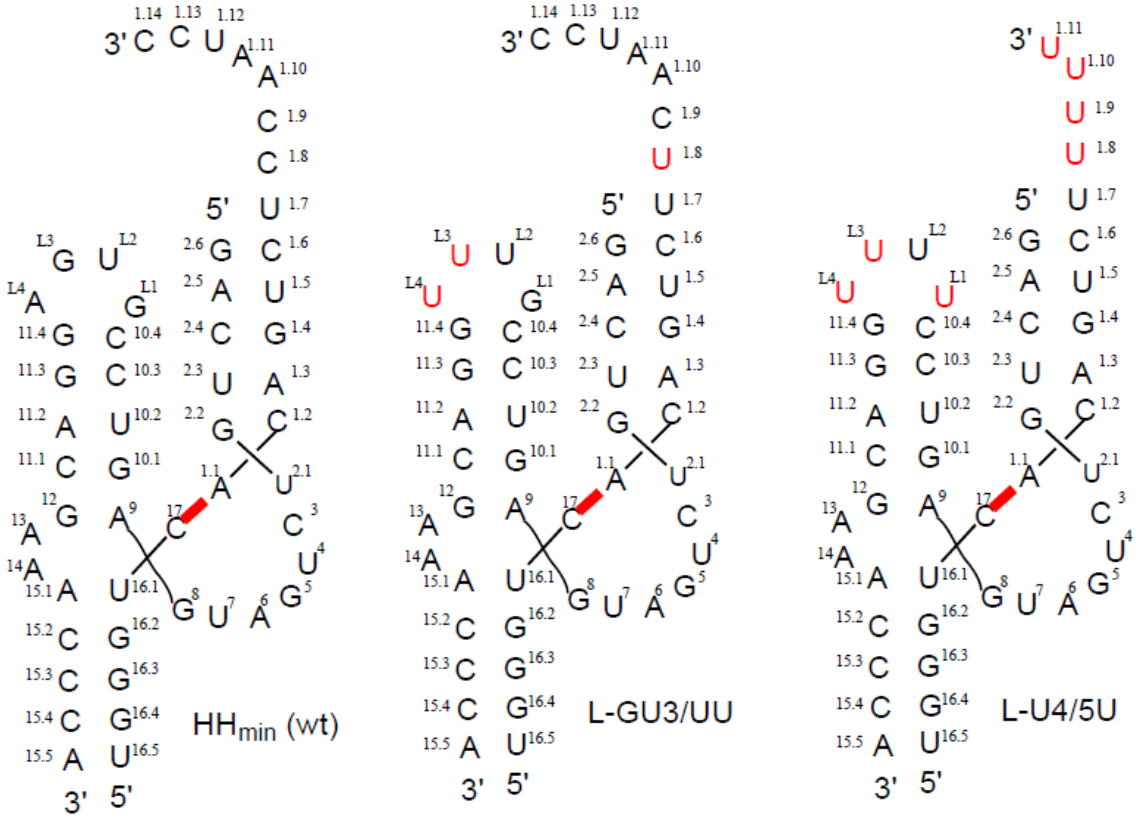


Figure S1.1 Secondary structure representation of the binary HHRs. Scissile bond shown in red between C17 and A1.1. Modified bases in the loop and stem I shown in red.

S1.1b) Transcription.

RNA constructs were made via T7 runoff transcription. RNA transcription buffer 10x: 1M HEPES-KOH pH 7.5, 100 mM $MgCl_2$, 20 mM Spermidine-HCl, 400 mM DTT. Transcription was performed in the following concentrations: 1x Buffer with an additional 10 mM DTT, 10 mM $MgCl_2$, 20 mM rNTP, 7.5% glycerol, 350 nM DNA template, and 2 µL of in house produced T7 polymerase stock (per 100 µl of transcription reaction solution, conditions subject to optimization based on sequences). Transcription was carried out at 37 °C for 2 hours, and then quenched with 1.5 equivalents of EDTA and an equal volume of 2x TBE/urea loading buffer. Samples were heated at 95 °C for 5 minutes, cooled to room temperature, and loaded onto acrylamide (19:1 acrylamide:bisacrylamide) denaturing gel (8.3cm X 7.3cm X 1.5mm) with 8M urea as the denaturant. The RNA was visualized using UV shadowing and desired gel bands cut, crushed through a syringe and soaked in water overnight with agitation at room temperature. The sample was centrifuged, supernatant removed, and gel discarded. The RNA was precipitated from the supernatant by addition of 10% volume of 5M NH_4OAc pH 5.2 and 2.5 volume equivalents of 200 proof ethanol, and incubation at -20 °C for at least 2 hrs. RNA was centrifuged down at high speed for 10 minutes at 4 °C and the pellet was washed with 500 µl of ice-cold 70% ethanol in water and centrifuged again at high speed for 10 minutes at 4 °C. RNA

pellets were then dried in a speed-vac at room temperature for 20 minutes and suspended in water and stored at -20 °C. Stock concentrations were determined by UV-vis spectroscopy, measuring absorbance at 260 nm. RNA purity was checked on a denaturing gel and visualized using SYBR gold staining.

S1.2. UV-melting.

UV-melting curves were measured on a Cary-100 UV-vis spectrophotometer equipped with an air-circulating temperature controller. All measurements were carried out with a temperature change rate of 1 °C/min and monitored at 260 nm. All samples are freshly annealed in 1X PBS buffer (pH=7.4) before measurements. Concentration of DNAs were 2 μM while the small molecule was held at 20 μM unless otherwise noted.

S1.3. Circular Dichroism(CD) spectroscopy.

CD spectra were obtained from a Jasco J815 Circular Dichroism Spectrometer equipped with a Peltier device and a water circulator. All measurements were taken at 25 °C in a Hellma quartz cell (1 mm path length) from 320-220 nm with data intervals of 0.5 nm, a bandwidth of 1 nm and D.I.T. of 2 s. For each sample three scans were collected, averaged together, and blank corrected. All samples were freshly annealed in 1X PBS buffer (pH=7.4) before measurements. DNA concentrations for all samples are 5 μM.

S1.4. Isothermal Titration Calorimetry (ITC).

ITC experiments were performed on a Nano ITC (TA instruments). DNA samples were diluted to 10 μM concentration in 1X PBS buffer (pH=7.4). Tren derivatives were dissolved in 1X PBS to desired concentrations. Both samples were degassed and subjected to the ITC experiment immediately. The reservoir contained 300 μL of the DNA solution and the microsyringe contained 50 μL of the small molecule solution. The stirring rate was set at 400 rpm with an injection volume of 2.02 μL with 250 s between each injection.

S1.5. Differential Scanning Calorimetry (DSC).

DSC experiments were carried out on a Microcalorimeter VP-DSC. Samples concentrations were set at 25 μM DNA and 250 μM tren derivatives in 1X PBS buffer (pH=7.4). Samples were scanned from 25 °C to 90 °C with 60 °C/h scanning rate, 16 s filtering period, and low feedback. A solution of 1X PBS was used as reference. Background data was collected with only 25 μM DNA in the sample cell and was subtracted from all DNA/tren sample traces.

S1.6. Fluorescence quenching assay.

FAM-$T_{10}C_4T_{10}$-Dab DNA, end-labeled with fluorescein (FAM) and DABCYL (4-(4'-dimethylaminophenylazo) benzoic acid) at the 5' and 3' ends, respectively, was purchased from IDT. DNA stock solutions were made by mixing 95 μM $dT_{10}C_4T_{10}$ and 5 μM FAM-$T_{10}C_4T_{10}$-Dab, due to the limited amount of FAM-$T_{10}C_4T_{10}$-Dab available. Samples were prepared by annealing 50 μM DNA stock and the tren derivatives (0-2 mM) in 1X PBS buffer prior to the experiment. Fluorescence of each sample was measured on a Thermo Fisher Nanodrop 3300.

S1.7. Fluorescence anisotropy.

A series of samples were made by mixing various concentrations of 12-T_n-12 (n=2 or 4) with a constant concentration of FITC labelled tren derivatives (100 nM) in 1X PBS. All samples were annealed at 95 °C for 5 min. Experiments were carried out on a Molecular Devices SpectraMax M5 instrument with excitation wavelength at 495 nm and emission monitored at 520 nm. Fluorescence anisotropy was converted into complex concentration using equation 1[1]:

$$[complex] = (\frac{FA - FAmin}{FAmax - FAmin}) * [tren]$$

FA, FA_{max}, FA_{min} correspond to current fluorescence anisotropy, maximum fluorescence anisotropy (FITC labelled tren derivatives fully bound to DNA) and minimum fluorescence anisotropy (FITC labelled tren derivatives alone), respectively. [complex] indicates the current complex concentration, [tren] indicates the concentration of FITC labelled tren derivatives (100 nM).

The concentration of the complex was plotted against total DNA concentration in each sample, and the result fitted to equation 2[2]

$$[complex] = (Kd + [DNA] + [tren])/2 - (\sqrt{(Kd + [DNA] + [tren])^2 - 4 * [DNA] * [tren]}\ )/2$$

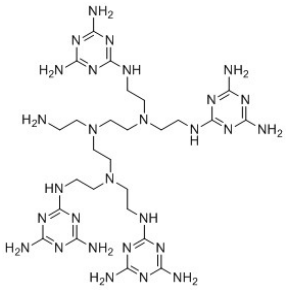
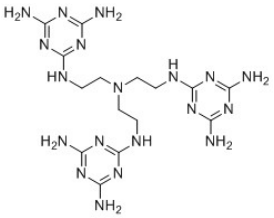
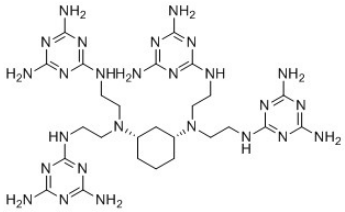
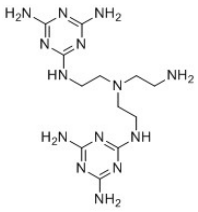
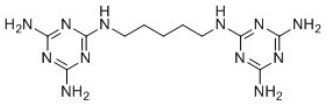
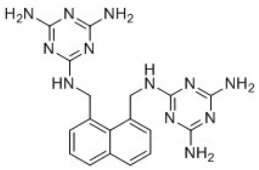
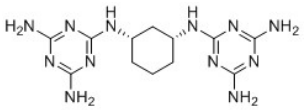
S1.8. EMSA.

A series of samples were made by mixing various concentrations of DNA duplexes and constant concentration of Cy5-t2M (1μM) in 1X PBS. All samples were incubated at room temperature for 30 min and then subjected to electrophoresis in a 15% TBE buffered native acrylamide gel at 120 V on ice. The gel was then scanned using Typhoon FLA 9500 (GE Healthcare).

S1.9. Additional scaffolds tested for DNA binding.

Alternate scaffolds based on the tren scaffold were tested for DNA binding. These compounds (Table S1.1) exhibited similar or diminished DNA binding as judged by preliminary thermal denaturation studies with $dT_{10}C_4T_{10}$ DNA. With the exception of t2M and t4M, these compounds were not studied in further detail. Compounds with similar thermal stability as t3M and t2M (**10** & **11**) were also less versatile scaffolds in that additional functionalization of the scaffold was not straightforward. Regardless, these preliminary data indicate where the tren design is corroborated (similar atom spacing between base triples in **10** and **11**) as well as the limits of this design. For instance, compounds **8** and **9** exhibit weak or undetectable thermal transitions, possibly due to increased steric interactions upon rigidification of the scaffold.

Table S1.1. DNA complexation data.[a]

Name	Structure	T_m with $dT_{10}C_4T_{10}$
t4M, **6**		66°C
t3M, **1**		47°C
10		46°C
t2M, **2a**		35°C (Boc t2M)
11		34°C
8		17°C
9		Not detected

[a] T_m is measured with solution concentrations of 2 μM DNA and 20 μM synthetic compound in 1X PBS.

S2. Additional data.

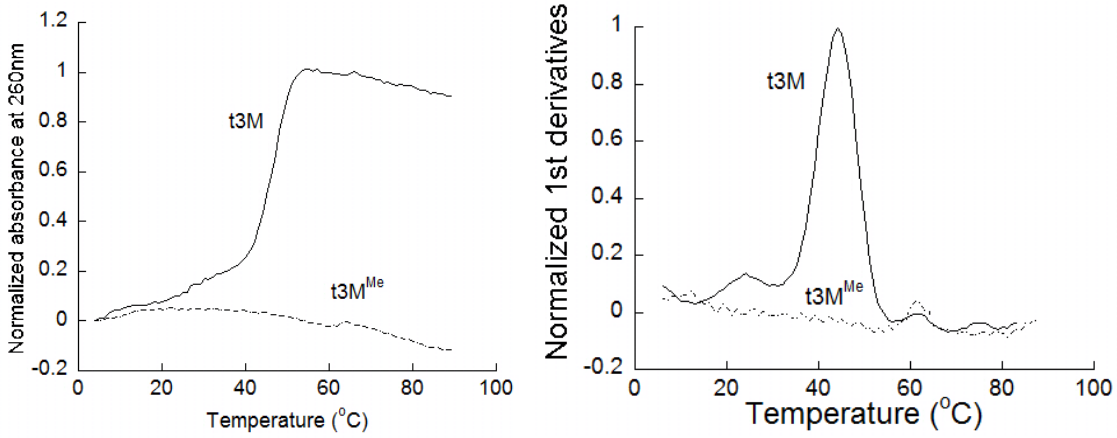


Figure S2.1 UV-melting of t3M and control with $dT_{10}C_4T_{10}$. Samples were prepared in 1X PBS. DNA concentrations were 2 μM. Tren derivatives concentrations were 20 μM. (Left) UV-melting traces show the change in absorbance at 260 nm with respect to temperature. (Right) First derivatives of the UV-melting traces with respect to temperature.

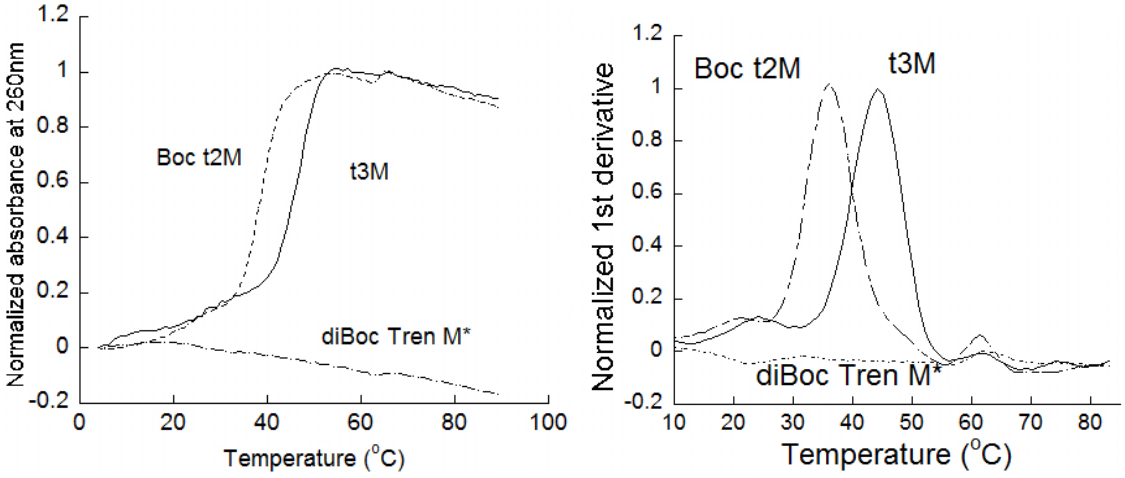


Figure S2.2 UV-melting of tren derivatives with $dT_{10}C_4T_{10}$. Samples were prepared in 1X PBS. DNA concentrations were 2 μM. Tren derivatives concentrations were 20 μM. (Left) UV-melting traces show the change in absorbance at 260 nm with respect to temperature. (Right) First derivatives of the UV-melting traces with respect to temperature.

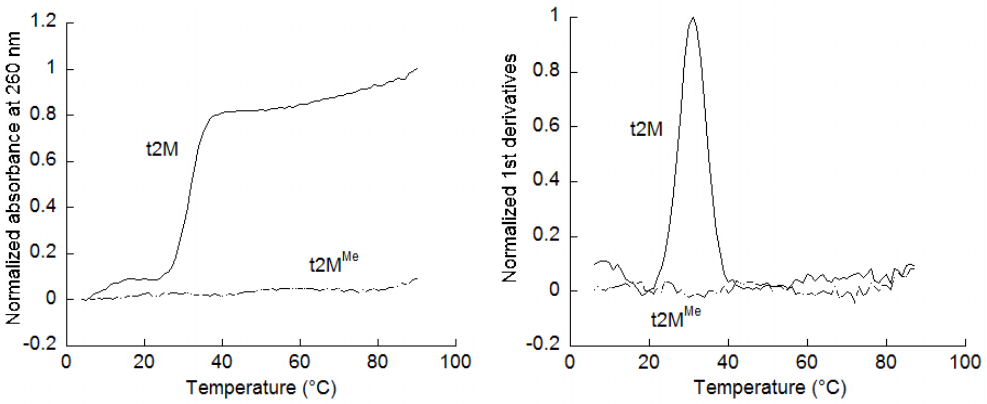


Figure S2.3 UV-melting of t2M and control with $dT_{10}C_4T_{10}$. Samples were prepared in 1X PBS. DNA concentrations were 2 μM. Tren derivatives concentrations were 20 μM. (Left) UV-melting traces show the change in absorbance at 260 nm with respect to temperature. (Right) First derivatives of the UV-melting traces with respect to temperature.

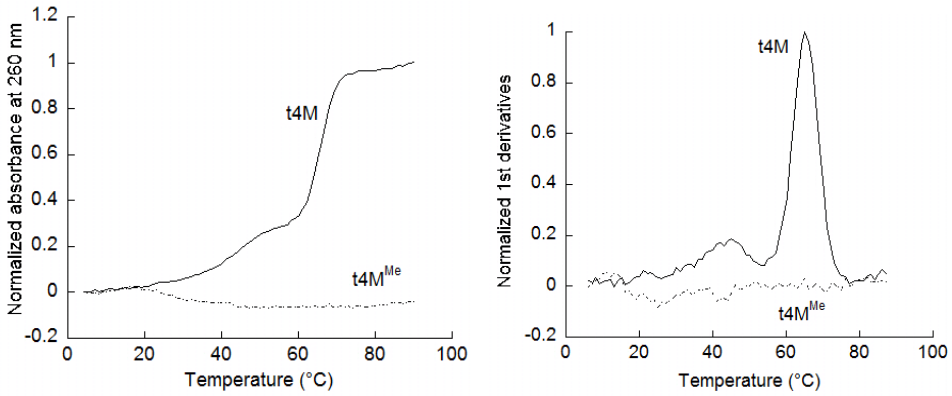


Figure S2.4 UV-melting of t4M and control with $dT_{10}C_4T_{10}$. Samples were prepared in 1X PBS. DNA concentrations were 2 μM. Tren derivatives concentrations were 10 μM. (Left) UV-melting traces show the change in absorbance at 260 nm with respect to temperature. (Right) First derivatives of the UV-melting traces with respect to temperature.

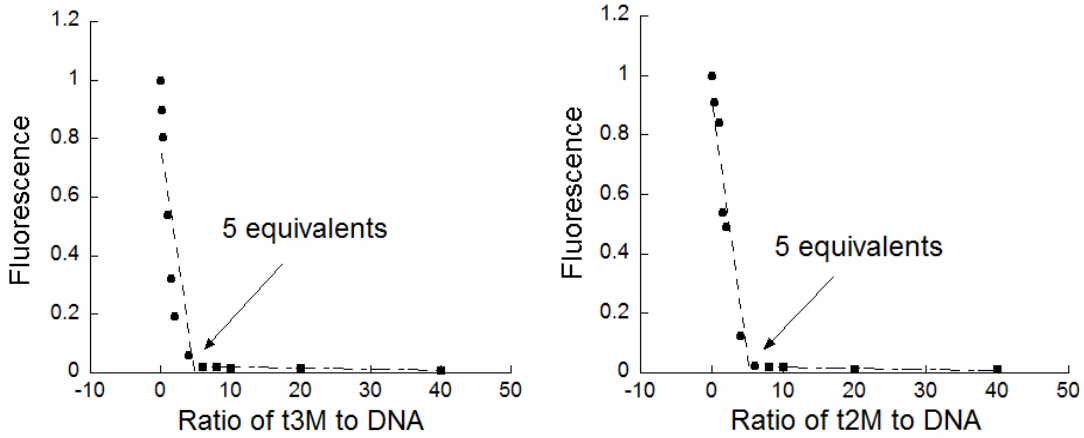


Figure S2.5 Job plots of molecular beacon fluorescence quenching with t3M and t2M. Various amounts of tren derivatives (0-2 mM) were titrated into the 50 μM DNA solution of 19:1 $dT_{10}C_4T_{10}$: FAM-$T_{10}C_4T_{10}$-Dab in 1X PBS. Fluorescence was measured on Thermo Fischer Nanodrop 3300.

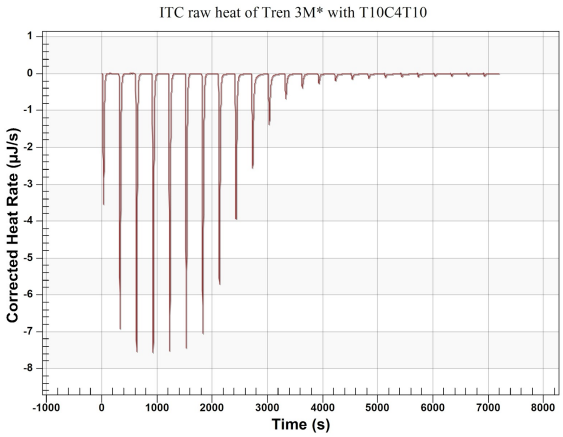


Figure S2.6 The above trace shows the experimental ITC raw heat of t3M (600 µM, 50µL, 1X PBS) injected into $dT_{10}C_4T_{10}$ (10µM, 300µL, 1X PBS).

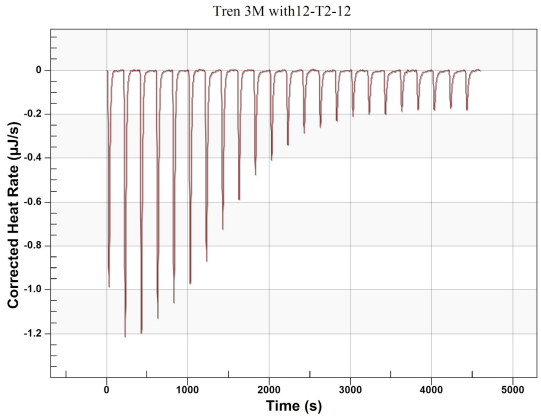


Figure S2.7 The above trace shows the experimental ITC raw heat of t3M (200 µM, 50µL, 1X PBS) injected into 12-T_2-12 (20 µM, 300 µL, 1X PBS).

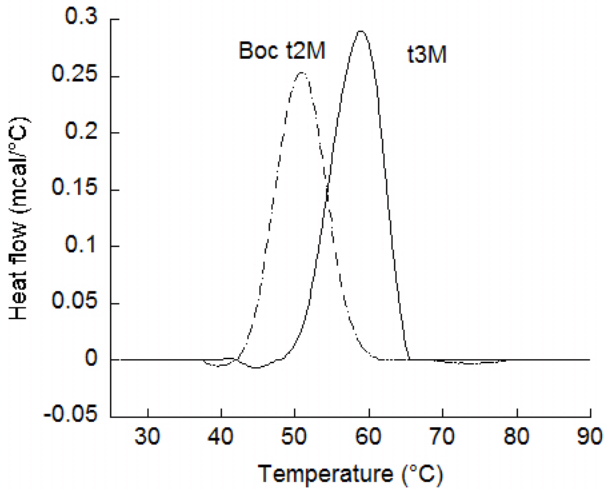


Figure S2.8 DSC curves of 25 µM $dT_{10}C_4T_{10}$ with Boc t2M or t3M. Tren derivatives concentration was 250 µM. Samples were prepared in 1X PBS buffer (pH=7.4) and scanned from 25 °C to 90 °C with 60 °C/h scanning rate, 16 s filtering period, and low feedback.

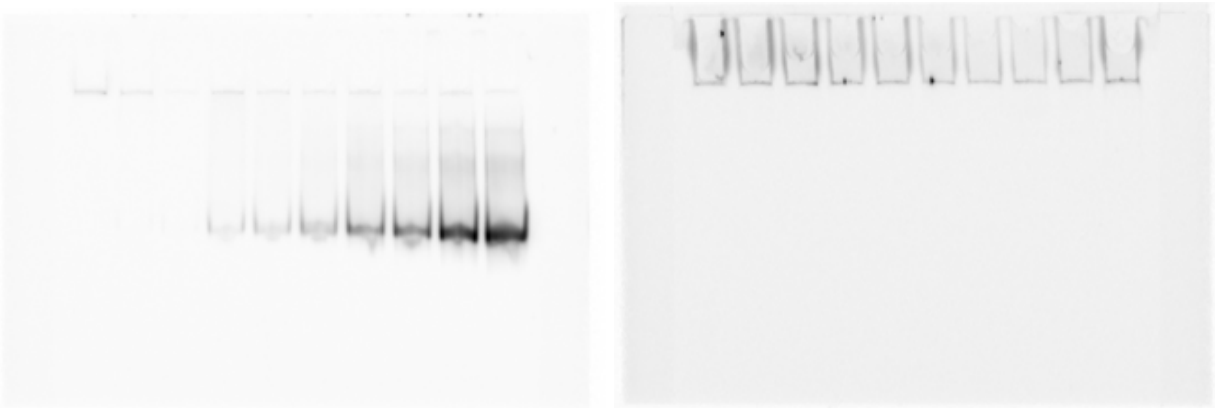


Figure S2.9 Gel shift of Cy5-t2M with 12-T_2-12 (left) and 12-T_0-4 (right). Cy5-t2M concentration was 1 μM in each lane, while the DNA concentrations were 0, 0.2, 0.5, 1, 2, 5,10, 20, 50, 100 μM from left to right in each gel. The bands in the left gel indicate the stained DNA Cy5-t2M complex.

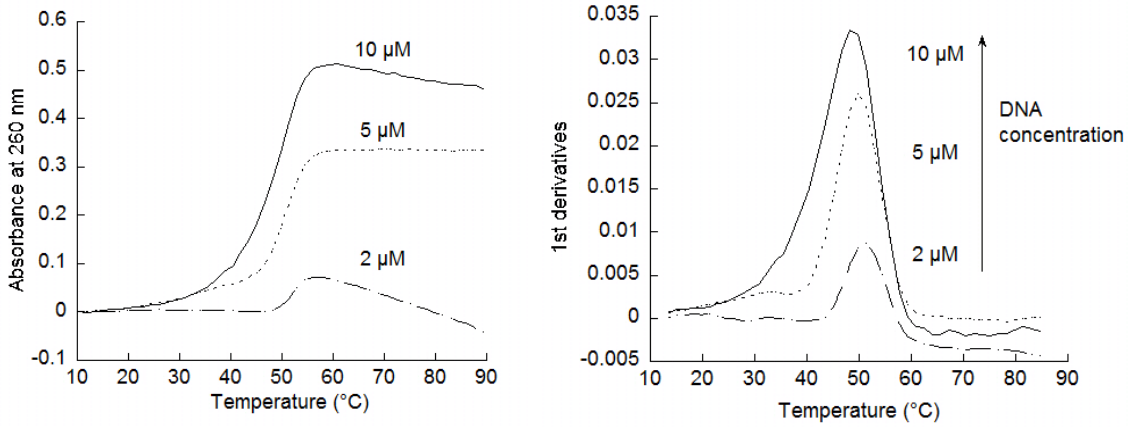
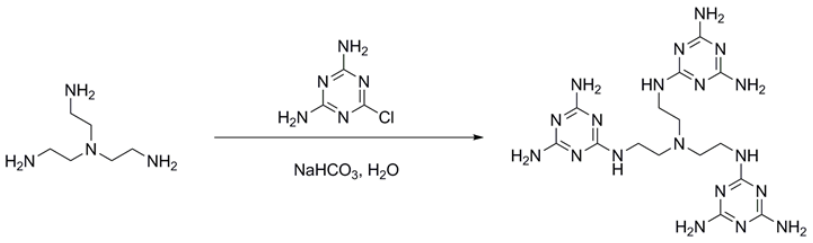


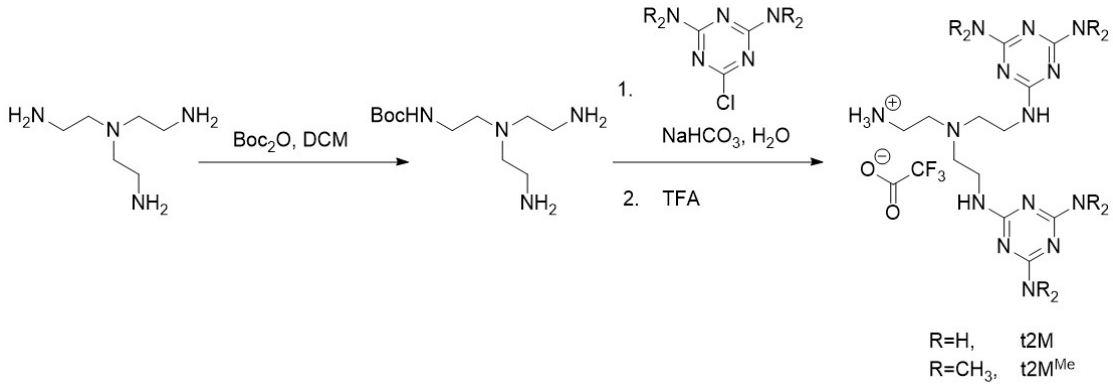
Figure S2.10 UV-melting of 50 μM t3M and various concentrations of $dT_{10}C_4T_{10}$. Samples were annealed at 95 °C in 1X PBS right before the experiment. (Left) UV-melting traces show the change in absorbance at 260 nm with respect to temperature. (Right) First derivatives of the UV-melting traces with respect to temperature.

S3. Synthetic procedures.



Scheme S3.1 Synthesis of t3M.

*N^2-(2-(bis(2-((4,6-diamino-1,3,5-triazin-2-yl)amino)ethyl)amino)ethyl)-1,3,5-triazine-2,4,6-triamin e (t3M, **1**).* (Scheme S3.1)
Tris(2-aminoethyl)amine (0.75 mL, 5 mmol) was dissolved in 30 mL H_2O, followed by addition of diaminochlorotriazine (4.36 g, 30 mmol) and $NaHCO_3$(1.5 g, 18 mmol). The reaction was heated to 85 °C and left to stir overnight. After cooling down to room temperature, the solution was filtered, the solid was washed with water twice, and dried under vacuum (1.63 g, 73%). The crude solid was further purified by prep HPLC using gradient of 0% to 15% acetonitrile over 40 minutes. ^{1}H NMR: 3.68 (6H, t); 3.88 (6H, t); ^{13}C NMR: 162.84; 159.11; 156.55; 52.61; 35.49; ESI: mass calculated$[M+H]^+$=474.2770; $[M+2H]^{2+}$=237.6421; mass observed: 474.2788; 237.6426.



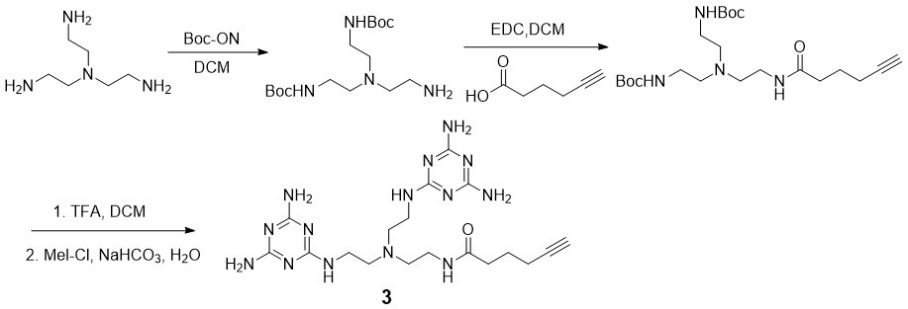
Scheme S3.2 Synthesis of t2M and $t2M^{Me}$.

2-(tert-butoxylcarbonylamino)ethyl-bis-[(2-aminoethyl)]amine. (Scheme S3.2)
Tris(2-aminoethyl)amine (6 mL, 40 mmol) was dissolved in 50 mL DCM and cooled in an ice bath. A solution of Boc anhydride (1.09 g, 5 mmol) in 20 mL DCM was added dropwise, followed by dropwise addition of triethylamine (0.7 mL, 5 mmol) in 20 mL DCM. The reaction was warmed up to room temperature slowly and stirred overnight. After removal of the solvent under reduced pressure, the crude oil was resuspended in 50 mL H_2O and extracted with DCM (50 mL x 5). The organic phase was dried over Na_2SO_4 and solvent removed under reduced pressure. Chromatographic purification of the crude product (DCM:MeOH=5:1, 2% concentrated ammonium hydroxide) yielded 700 mg (57%) of a light yellow oil.

t2M, **2a**. (Scheme S3.2)
N^2-(2-((2-aminoethyl)(2-((4,6-diamino-1,3,5-triazin-2-yl)amino)ethyl)amino)ethyl)-1,3,5-triazine-2, 4,6-triamine. 6-chloro-2,4-diamino-1,3,5-triazine(550 mg, 3.78 mmol) and $NaHCO_3$ (500 mg, 5.95 mmol) were added into a 40 mL H_2O solution of mono-Boc protected tren (220 mg, 0.89 mmol). The slurry was heated to 85 °C and reacted overnight. The reaction was then cooled to room temperature and filtered. The solid was washed twice with water and dried under vacuum. The crude product was then purified by silica gel chromatography using DCM:MeOH:conc NH_4OH=80:20:2 to yield Boc-t2M as a white solid (90 mg, 22%). ^{1}H NMR: 1.32 (9H, s); 3.45

(8H, m); 3.62 (4H, m); ^{13}C NMR: 27.52; 35.39; 35.97 54.04; 54.68; 81.82; 158.23; 164.98; 165.71; ESI: mass calculated[M+H]$^{+}$=465.2905; mass observed: 465.2914.
Boc t2M was dissolved in trifluoroacetic acid (TFA) and reacted at room temperature for 10 min. The TFA was evaporated under N_2 flow, the residue was dissolved in H_2O, and solution lyophilized to obtain the TFA salt of t2M. ^{1}H NMR: 3.43 (2H, m); 3.52 (4H, m); 3.66 (2H, m); 3.74 (4H, m); ^{13}C NMR: 166.73; 162.54; 159.25; 156.62; 52.76; 49.95; 35.58; 33.43; ESI: mass calculated[M+H]$^{+}$=365.2381; [M+2H]$^{2+}$=183.1227; mass observed: 365.2368; 183.1238.

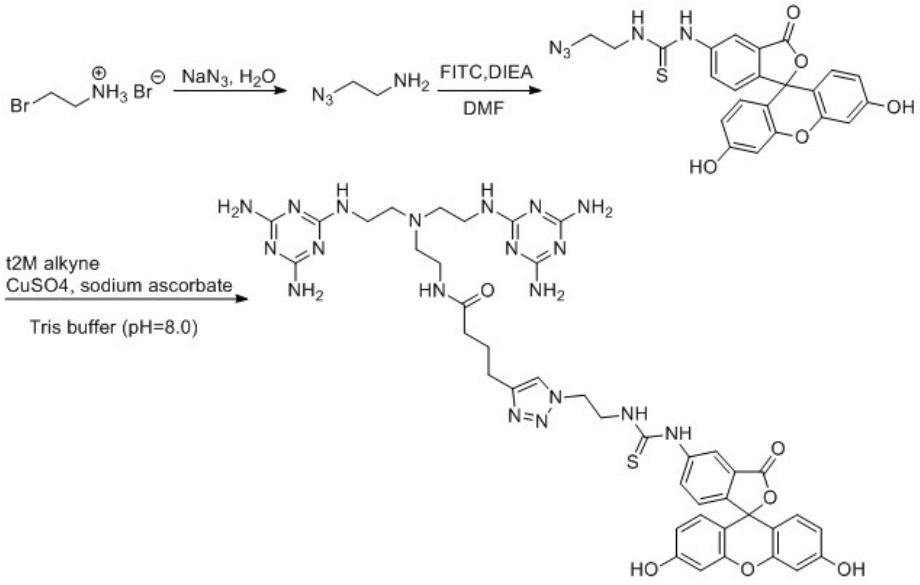
t2M^{Me}, **2b**.
N^{2}-(2-((2-aminoethyl)(2-((4,6-bis(dimethylamino)-1,3,5-triazin-2-yl)amino)ethyl)amino)ethyl)-N^{4},N^{4},N^{6},N^{6}-tetramethyl-1,3,5-triazine-2,4,6-triamine. (Scheme S3.2)
6-chloro-N^{2},N^{2},N^{4},N^{4}-tetramethyl-2,4-diamino-1,3,5-triazine (80 mg, 0.4 mmol) and $NaHCO_3$ (84 mg, 1 mmol) were added into 10 mL 1,4-dioxane:H_2O=1:1 solution of mono Boc-protected tren (40 mg, 0.162 mmol). The slurry was heated to 85°C and reacted overnight. The reaction was then cooled to RT and extracted with DCM twice. The organic layer was then dried and condensed to obtain a crude mixture which was purified by silica gel chromatography using 2% MeOH in DCM with 2% concentrated ammonia in MeOH. The product was obtained as a white solid (45 mg, 48.4%). ^{1}H NMR: 1.41 (9H, s); 2.61 (6H, m); 3.05 (26H, m); 3.37 (4H, m); 5.29 (2H, t); 5.55 (1H, t); ^{13}C NMR: 166.44; 165.92; 156.38; 78.99; 53.69; 38.93; 36.07; 28.54 ; ESI: mass calculated: [M+H]$^{+}$=577.4157; [M+2H]$^{2+}$=289.2115; mass observed:577.4148; 289.2105.
Boc t2M^{Me} was dissolved in TFA and reacted at room temperature for 10 min. The TFA was evaporated under N_2 flow, the residue was dissolved in H_2O and lyophilized to obtain the TFA salt of t2M^{Me}. ^{1}H NMR: 3.04 (24H, d); 3.33 (2H, m); 3.35 (6H, m); 3.78 (4H, m); ^{13}C NMR: 162.25; 156.47; 156.62; 52.73; 51.07; 37.38; ESI: mass calculated[M+H]$^{+}$=477.3633; [M+2H]$^{2+}$=239.1853; mass observed: 477.3615; 239.1857.



Scheme S3.3 Synthesis of t2M alkyne.

t2M alkyne (**3**). (Scheme S3.3)
N-(2-(bis(2-((4,6-diamino-1,3,5-triazin-2-yl)amino)ethyl)amino)ethyl)- hex-5-ynamide.
Di-tert-butyl (((2-aminoethyl)azanediyl)bis(ethane-2,1-diyl))dicarbamate (di-Boc tren) was synthesized based on literature procedure.[3] 5-Hexynoic acid (0.1 mL, 0.97 mmol) was mixed with 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (139 mg, 0.9 mmol) in DCM and reacted for 0.5 h. Di-Boc tren (175 mg, 0.5 mmol) was then added to the solution and reacted for 2h. The reaction was diluted to 50 mL DCM, washed with 30 mL 1N HCl (aq), 50 mL

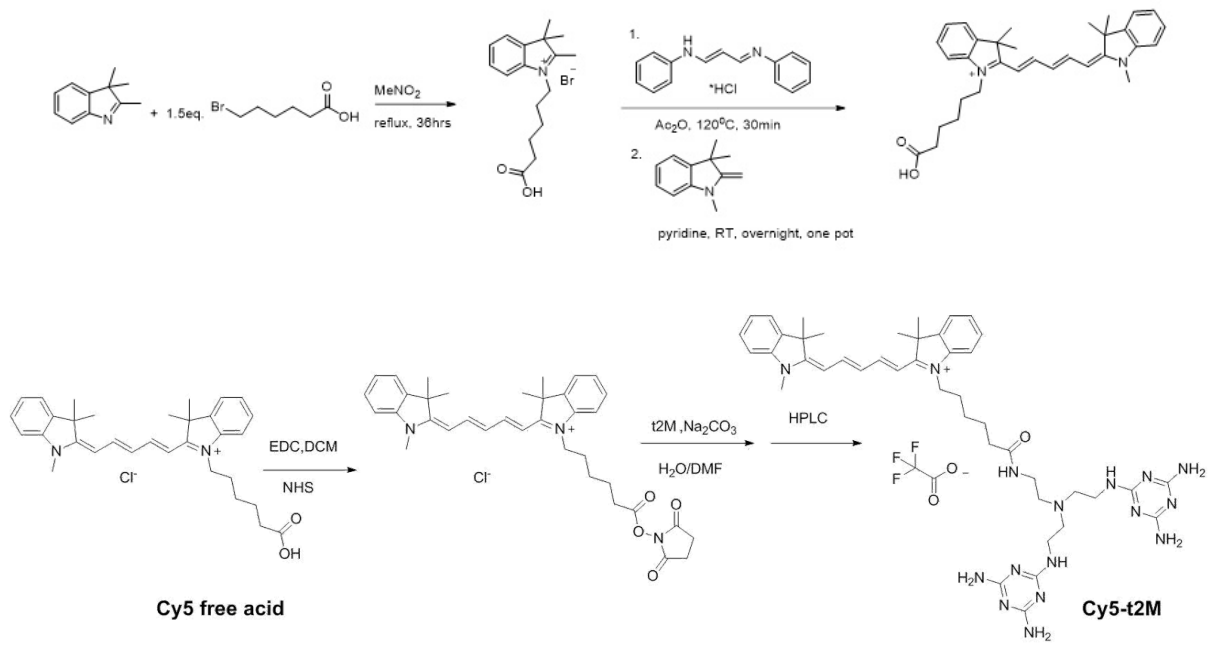
saturated $NaHCO_3$ (aq), and 50 mL brine. The organic layer was then dried and condensed to obtain the crude product which was purified on silica gel column with a DCM/MeOH gradient from 20:1 to 10:1 to obtain 150 mg of di-Boc tren alkyne as a white solid (68%). Di-Boc tren alkyne was dissolved in 10 mL of 1:1 DCM/TFA and reacted at room temperature for 30 min. The solution was condensed to syrup under a stream of N_2 before cold Et_2O was added to form a white precipitate. The precipitate was centrifuged down, washed with cold Et_2O and dried under vacuum to yield the TFA salt of tren alkyne. It was then dissolved in H_2O and adjusted to pH=9 with $NaHCO_3$. 6-chloro-2,4-diamino-1,3,5-triazine was then added to the solution and additional $NaHCO_3$ in order to keeping the pH at 9. The slurry was heated to 85 °C and reacted overnight. After cooling down to room temperature, a white precipitate formed which was centrifuged and washed with water to yield crude t2M alkyne **3**. It was further purified by prep HPLC using gradient of 0% to 20% acetonitrile over 40 minutes. ^{1}H NMR: 1.77 (2H, m); 2.24 (2H, t); 2.38 (3H, m. contains terminal alkyne proton); 3.56 (2H, t); 3.62 (6H, m); 3.85 (4H,t); ESI: mass calculated $[M+2H]^{2+}$=230.1436; mass observed: 230.1452.



Scheme S3.4 Synthesis of FITC-t2M.

Typical procedure for installation of fluorescein on tren derivatives.

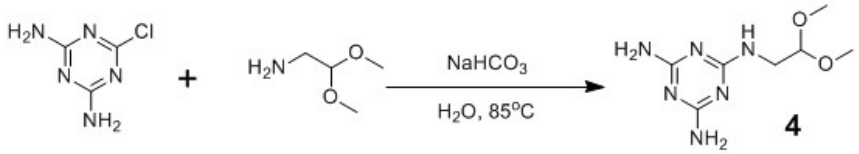
Fl-t2M (Scheme S3.4): FITC-N_3 was synthesized according to literature procedure.[4] To an aqueous solution of t2M alkyne (18 mM, 10 µL) was added FITC-N_3 in DMSO (300 mM, 10 µL) and 50 µL of 1 M Tris-Cl (pH=8.0). Then $CuSO_4$(0.1 M, 10 µL) and a sodium ascorbate(0.1 M, 20 µL) solution was added. The mixture was then reacted for 1 h at room temperature followed by quenching with the addition of 1N HCl. Product was purified by HPLC on C18 column to yield FITC-t2M (6.4 mM, 50 µL, 89%). ESI: mass calculated $[M+H]^+$= 934.3750; $[M+2H]^{2+}$=467.6911; $[M+3H]^{3+}$=312.1299; mass observed: 934.3610; 467.6890; 312.1285.



Scheme S3.5 Synthesis of Cy5-t2M.

Typical procedure for installation of Cy5 on tren derivatives.

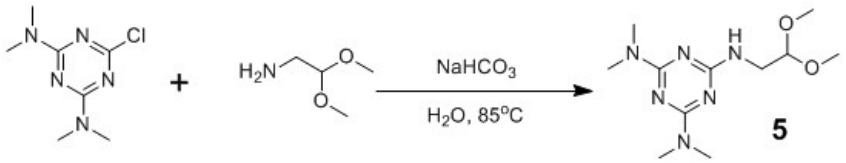
Cy5-t2M (Scheme S3.5): Cy5 free acid was synthesized according to literature procedure.[5] The free acid (15 mg) was then dissolved in 5 mL DCM and to this solution was added 7 mg of EDC and 8 mg of N-hydroxysuccinimide (NHS). The reaction was stirred at room temperature for 2 h. The reaction was then diluted in 50 mL DCM and washed with 20 mL 1N HCl and 20 mL brine. The organic layer was dried over Na_2SO_4 and condensed to yield crude product (12 mg). It was then dissolved in DMF to make a 15 mM solution, to which 18 mM aqueous solution of t2M was added. A 0.1 M Na_2CO_3 solution was used to adjust the pH to 9. The reaction mixture was stirred for 2 hrs, then purified by HPLC to obtain Cy5-t2M. ESI: mass calculated $[M+2H]^{2+}$=415.2641; mass observed: 415.2619.



Scheme S3.6 Synthesis of N^2-(2,2-dimethoxyethyl)-1,3,5-triazine-2,4,6-triamine.

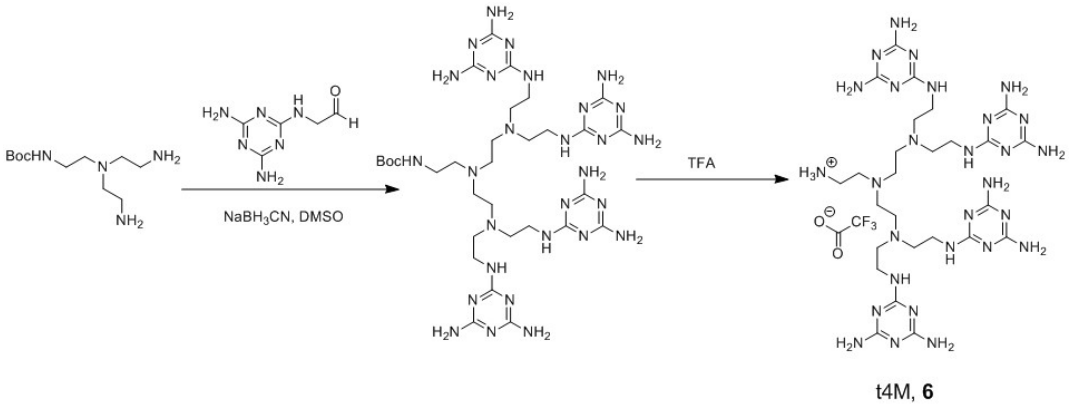
N^2-(2,2-dimethoxyethyl)-1,3,5-triazine-2,4,6-triamine (**4**). (Scheme S3.6) Aminoacetaldehyde dimethyl acetal (4 mL, 38.7 mmol) was dissolved in 30 mL H_2O at which point 6-chloro-2,4-diamino-1,3,5-triazine (4.35 g, 30 mmol) and $NaHCO_3$(2.8 g, 33 mmol) were added to the solution. The reaction was heated to 85 °C and stirred overnight. After cooling to RT, the reaction was filtered and the solid was washed with water twice. After drying under vacuum, N^2-(2,2-dimethoxyethyl)-1,3,5- triazine-2,4,6-triamine (**4**) was collected as a white solid (4.5 g, 70.1%) ^{1}H NMR: 3.26 (6H,s); 3.28 (2H, m); 4.45 (1H,t); 5.99-6.14 (4H, br); 6.40 (1H, t); ^{13}C

NMR: 41.47; 52.93; 102.04; 150.04; 166.37; ESI: mass calculated $[M+H]^+$=215.1251; mass observed: 215.1262. Deprotection of **4** in 1N HCl for 2 h at room temperature followed by drying to remove solvent in vacuo yielded melamine acetaldehyde as a white solid and was used without further purification.



Scheme S3.7 Synthesis of N^2-(2,2-dimethoxyethyl)-N^4,N^4,N^6,N^6-tetramethyl-1,3,5-triazine-2,4,6-triamine.

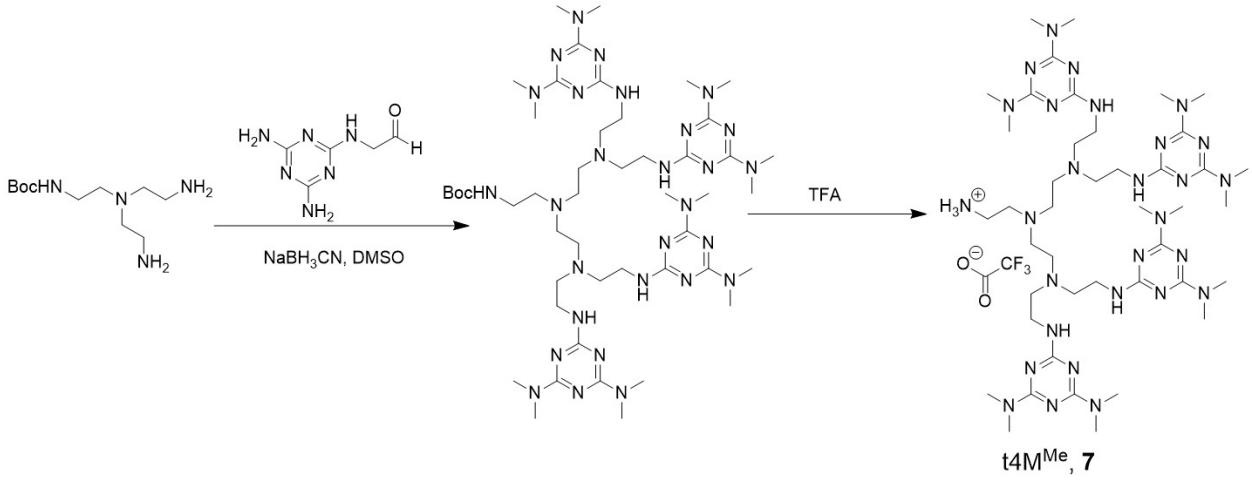
*N^2-(2,2-dimethoxyethyl)-N^4,N^4,N^6,N^6-tetramethyl-1,3,5-triazine-2,4,6-triamine (**5**).*(Scheme S3.7) Aminoacetaldehyde dimethyl acetal (1.64 mL, 15 mmol) was dissolved in 10 mL H_2O:1,4-dioxane=1:1 mixture followed by the addition of 6-chloro-N^2,N^2,N^4,N^4-tetramethyl-2,4-diamino-1,3,5-triazine (2.01 g, 10 mmol) and $NaHCO_3$ (2.8 g, 33 mmol). The reaction was heated to 85°C and stirred overnight. After cooling down, the reaction was filtered and the solid was washed with water twice. After drying under reduced pressure, **5** was collected as a white solid (2.1 g, 77.8%) ^{1}H NMR: 4.91 (1H, t); 4.45 (1H, t); 3.50 (2H, dd); 3.36 (6H, s); 3.05 (12H, s) ; ^{13}C NMR:; 166.12; 165.77; 103.18; 54.04; 42.25; 35.89; ESI: mass calculated $[M+H]^+$=271.1877; mass observed: 271.1876. Deprotection of **5** in 1N HCl for 2h at room temperature followed by solvent removal *in vacuo* yielded tetramethyl melamine acetaldehyde as white solid and was used without further purification.



Scheme S3.8 Synthesis of t4M.

t4M, (**6**). (Scheme S3.8) To a 1M DMSO solution of mono Boc-protected tren (15 mg, 0.061 mmol) was added two equivalents of melamine acetaldehyde as a 1 M DMSO solution and 2 equivalents of of $NaBH_3CN$ as a 1 M DMSO solution. After 2 hours an additional 2.5 equivalents of melamine acetaldehyde and $NaBH_3CN$ were added, both as 1 M solutions in

DMSO and reacted for another 2 hours. The reaction process was monitored by HPLC and MS. The reaction mixture was then precipitated, washed in DCM, and purified by HPLC to obtain Boc-t4M as a white solid (70% by HPLC). The solid was then dissolved in TFA and reacted at room temperature for 10min. The TFA was evaporated under N_2 flow and the residue was dissolved in H_2O and lyophilized to obtain the TFA salt of t4M. ^{1}H NMR: 2.86 (2H, t); 3.00-3.08 (6H, m); 3.43-3.52 (12H, m); 3.77 (8H, t); 3.78 (8H, t); ^{13}C NMR: 165.68; 54.11; 51.10; 49.94; 47.86; 36.34; 36.05; ESI: mass calculated: $[M+H]^+$=755.4846; $[M+2H]^{2+}$=378.2459; $[M+3H]^{3+}$=252.4997; $[M+4H]^{4+}$=189.6269. mass observed: 755.4799; 378.2437; 252.4988; 189.6269.



Scheme S3.9 Synthesis of t4M^{Me}.

t4M^{Me}, (**7**). (Scheme S3.9) To a 1 M DMSO solution of mono Boc-protected tren was added added two equivalents of tetramethyl melamine acetaldehyde as a 1 M DMSO solution and 2 equivalents of of $NaBH_3CN$ as a 1 M DMSO solution. After 2 hours an additional 2.5 equivalents of tetramethyl melamine acetaldehyde and $NaBH_3CN$ were added, both as 1 M solutions in DMSO and reacted for another 2 hours. The reaction mixture was then precipitated into 1 M NaOH solution, the solid was separated by centrifuge and washed with H_2O. After drying, the solid was purified on silica gel column with 2% MeOH in DCM and 1% NH_4OH (aq) to obtain 35 mg (53.8%) white solid. The solid was then dissolved in TFA and reacted at room temperature for 10 min. The TFA was evaporated under N_2 flow and the residue was dissolved in H_2O and lyophilized to obtain the TFA salt of t4M^{Me}. ^{1}H NMR: 2.77 (2H, t); 2.91-3.08 (56H, br); 3.31 (4H, t); 3.44 (8H, t); 3.78 (8H, t); ^{13}C NMR: 158.34; 156.46; 54.46; 53.85; 52.77; 49.92; 39.43; 39.13; 38.03; ESI: mass calculated: $[M+2H]^{2+}$=490.3711; $[M+3H]^{3+}$=327.2499; mass observed: 490.3701; 327.2495.

S4. Ribozyme cleavage data.

S4.1. U-(2,3) ribozyme.

A stock solution with U-(2,3) ribozyme (625 nM) and tren derivatives (12.5 μM) in 1.25X Tris-Cl buffer (pH=7.6) was annealed at 75 °C. An aqueous $MgCl_2$ solution was added to the annealed solution to initiate the reaction at 37 °C. The final concentrations were: 500 nM ribozyme, 10 μM tren derivatives and 10 mM Mg^{2+} in 1X Tris-Cl buffer (pH=7.6). Aliquots were taken at 0, 10, 45, 90, 150 min, quenched with urea-EDTA, and frozen immediately on dry ice. Samples were analyzed on 12% denaturing acrylamide gel and stained with SYBR® gold.

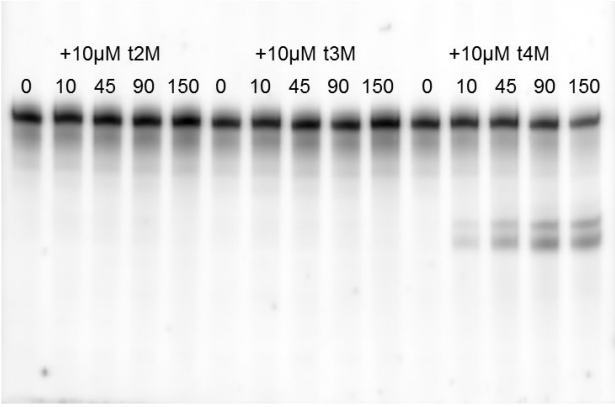


Figure S4.1 U-(2,3) ribozyme activity rescued by 10 μM tren derivatives, under 10 mM Mg^{2+} conditions. Top band indicates intact U-(2,3) ribozyme, bottom two bands indicate the cleavage products.

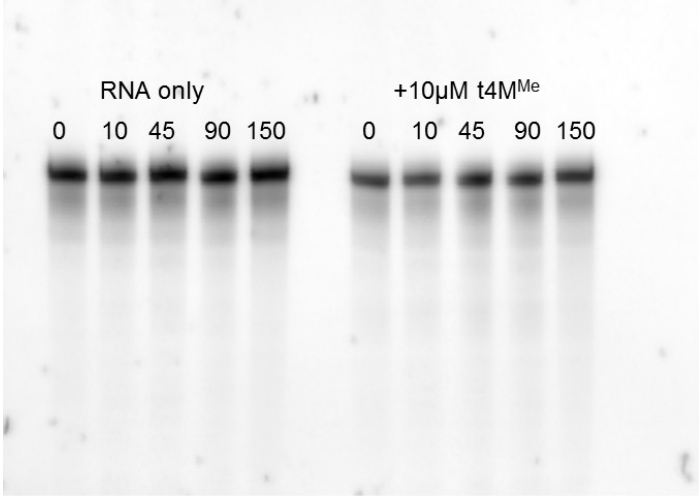


Figure S4.2 U-(2,3) ribozyme activity rescued by 10 μM tren derivatives, under 10 mM Mg^{2+} conditions. Top band indicates intact U-(2,3) ribozyme, no obvious cleavage products were observed.

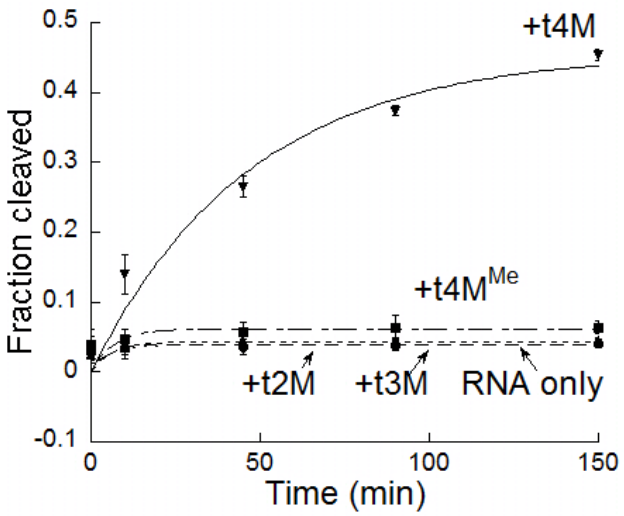


Figure S4.3 U-(2,3) ribozyme cleavage quantification, ribozyme activity rescued by 10 μM tren derivatives, under 10 mM Mg^{2+} conditions.

S4.2. S-U4n ribozymes.

A stock solution with S-U4n ribozyme (625 nM) and tren derivatives (12.5 μM) in 1.25X Tris-Cl buffer (pH=7.6) was heated at 75 °C for 2 min and equilibrated at 37 °C for 5 min. An aqueous $MgCl_2$ solution was added to the annealed solution to initiate the reaction at 37 °C. The final concentrations were: 500 nM ribozyme, 10 μM tren derivatives and 0.1 mM Mg^{2+} in 1X Tris-Cl buffer (pH=7.6). Aliquots were taken at 0, 5, 15, 30, 60, 90 min, quenched with urea-EDTA, and frozen immediately on dry ice. Samples were analyzed on 12% denaturing acrylamide gel and stained with SYBR® gold.

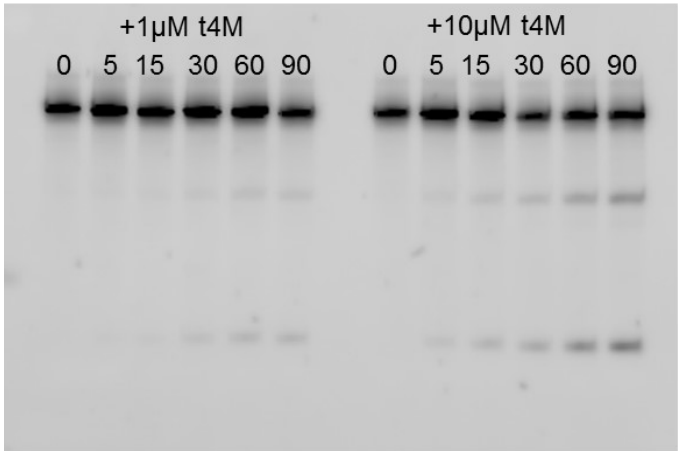


Figure S4.4 S-U4n ribozyme rescued by t4M, under 0.1 mM Mg^{2+} conditions. Top band indicates intact S-U4n ribozyme, middle band indicates the tRNA cleavage product, bottom band indicates the ribozyme cleavage product.

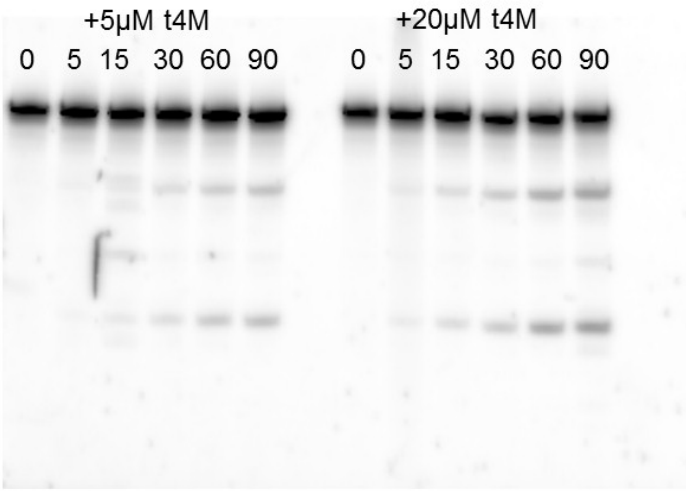


Figure S4.5 S-U4n ribozyme rescued by t4M, under 0.1 mM Mg^{2+} conditions. Top band indicates intact S-U4n ribozyme, middle band indicates the tRNA cleavage product, bottom band indicates the ribozyme cleavage product.

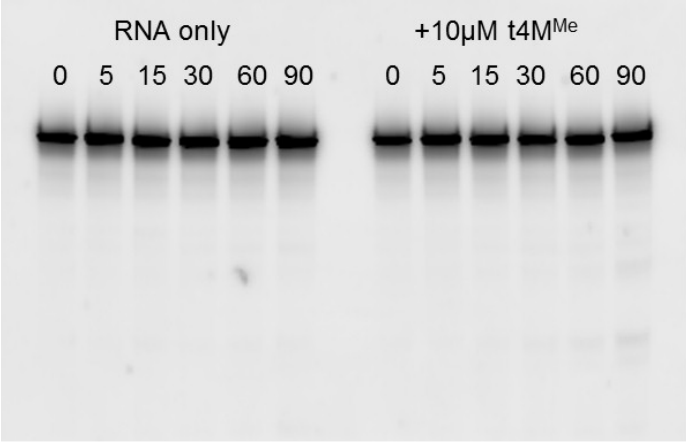


Figure S4.6 S-U4n ribozyme cleavage with methylated t4M control molecule, under 0.1 mM Mg^{2+} conditions. Top band indicates intact S-U4n ribozyme, middle band indicates the tRNA cleavage product, bottom band indicates the ribozyme cleavage product.

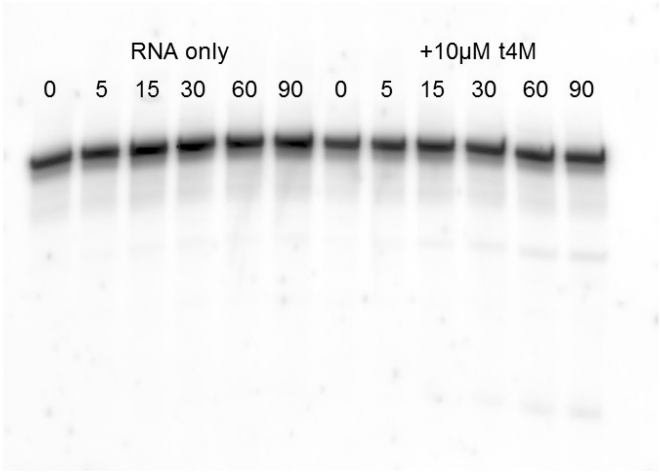


Figure S4.7 S-U4n-2bp ribozyme rescued by t4M, under 0.1 mM Mg^{2+} conditions. Top band indicates intact S-U4n-2bp ribozyme, middle band indicates the tRNA cleavage product, bottom band indicates the ribozyme cleavage product.

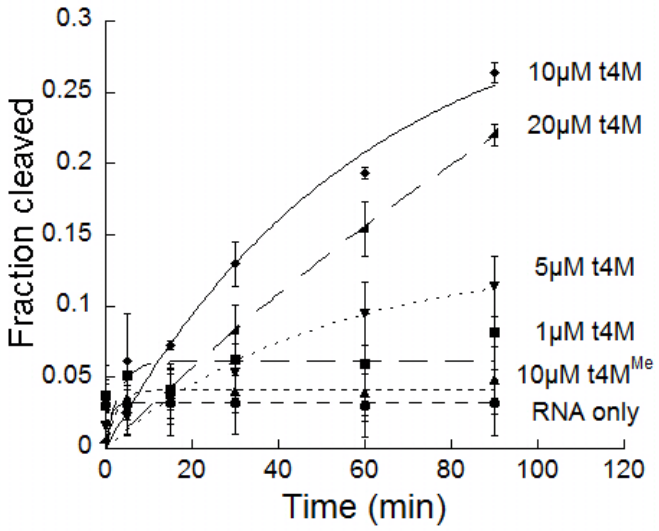


Figure S4.8 S-U4n ribozyme cleavage data quantification, with various concentrations of t4M, under 0.1 mM Mg^{2+} conditions.

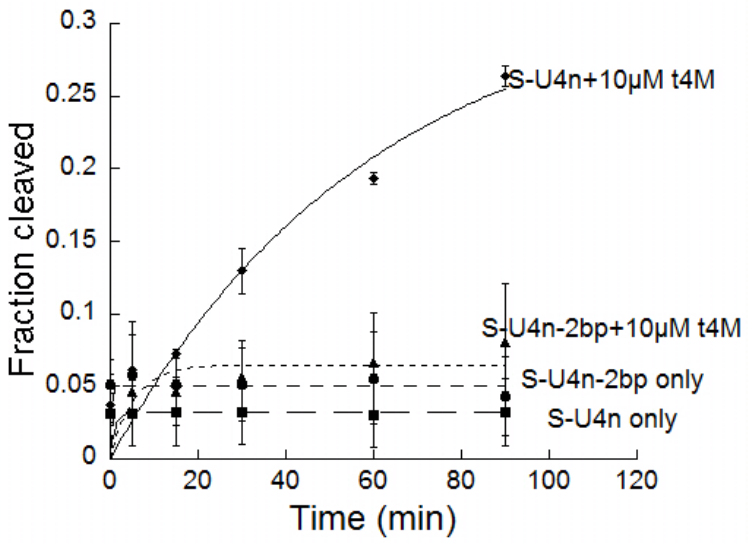


Figure S4.9 S-U4n and S-U4n-2bp ribozyme cleavage data quantification, with or without t4M, under 0.1 mM Mg^{2+} conditions.

S4.3. Binary (Enzyme-Substrate) ribozyme complexes.

Samples for single turnover experiments were prepared by making a stock solution with concentrations of the following species: 5 μM of enzyme strand, 400 nM of the Cy3 labeled substrate strand, and 10 μM tren derivatives in 1.25X Tris-Cl buffer (pH=7.6). The mixture was heated at 75 °C for 2 min and equilibrated at 27 °C for 10 min. $MgCl_2$ was then added to initiate the reaction at 27 °C. The final concentrations were: 4 μM enzyme strand, 320 nM Cy3 labeled substrate strand, 10 μM tren derivatives and 0.1 mM Mg^{2+} in 1X Tris-Cl buffer (pH=7.6). Aliquots were taken at 0, 15, 30, 45 s and 1, 2, 3, 5, 15, 30, 60 min, quenched with urea-EDTA, and frozen immediately on dry ice. Samples were analyzed on 20% denaturing acrylamide gel.

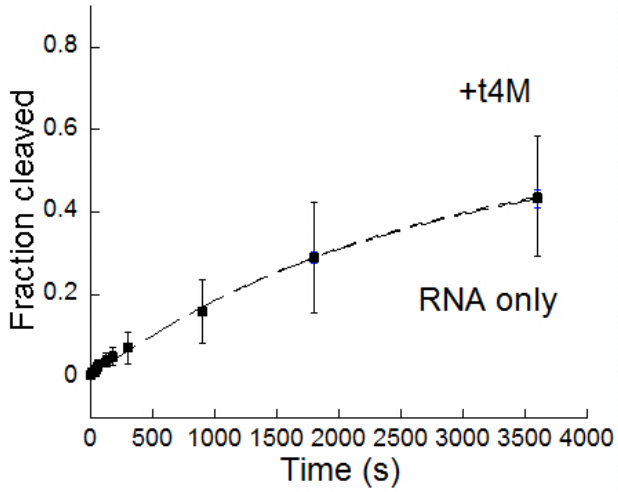


Figure S4.10 Binary ribozyme cleavage data quantification, L-U4/wt activity rescued by t4M, under 0.1 mM Mg^{2+} conditions.

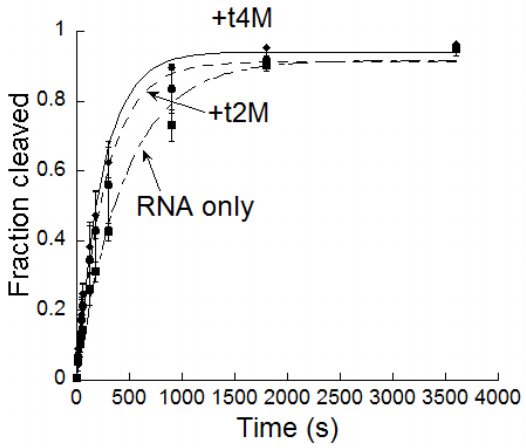


Figure S4.11 Binary ribozyme cleavage data quantification, L-U4/UU activity rescued by tren tren derivatives, under 0.1 mM Mg^{2+} conditions.

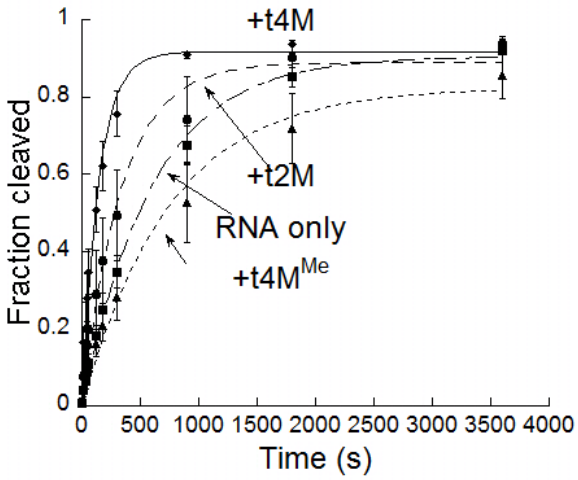


Figure S4.12 Binary ribozyme cleavage data quantification, L-U4/5U activity rescued by tren derivatives, under 0.1 mM Mg^{2+} conditions.

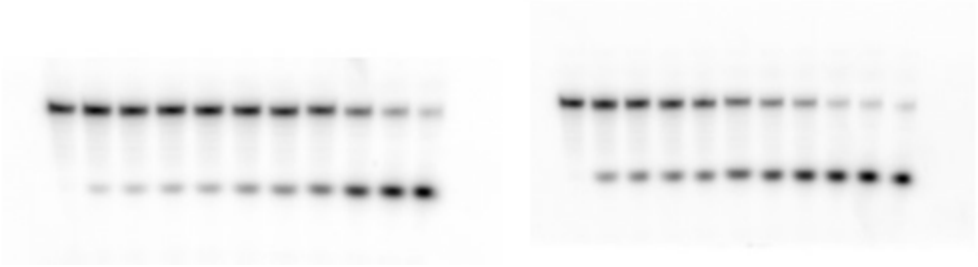


Figure S4.13 Representative gel image for binary ribozyme cleavage. Top band is the intact Cy3 labelled substrate strand RNA, bottom band indicates the cleavage product. (Left) L-U4/5U; (right) L-U4/5U with 10 μM t4M, under 0.1 mM Mg^{2+} conditions.

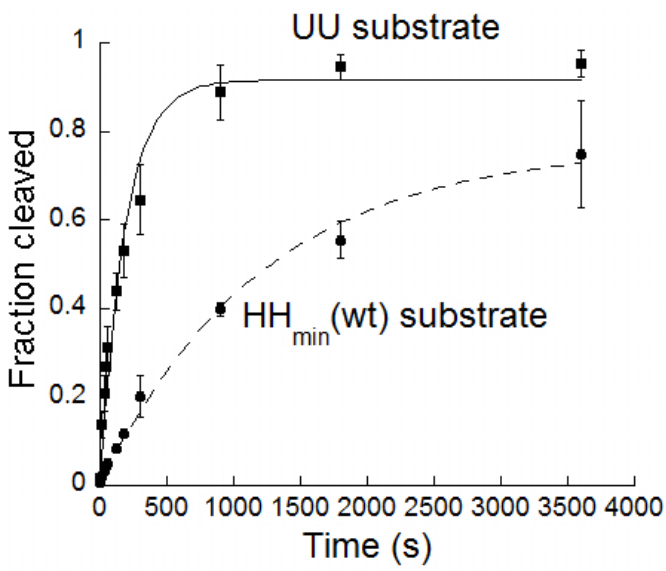


Figure S4.14 Binary ribozyme L-GU3 enzyme with HH_{min}(wt) and UU substrates (S1.1a)), under 0.1 mM Mg^{2+} conditions.

Table S4.1 Summary of cleavage rates of binary ribozymes.

Enzyme	Substrate	Tren derivative	[Mg^{2+}]	Cleavage rate
L-U4	wt	None	0.1 mM	0.023±0.003 min^{-1}
L-U4	wt	t4M	0.1 mM	0.023±0.003 min^{-1}
L-U4	UU	None	0.1 mM	0.133±0.012 min^{-1}
L-U4	UU	t2M	0.1 mM	0.217±0.015 min^{-1}
L-U4	UU	t4M	0.1 mM	0.245±0.014 min^{-1}
L-U4	5U	None	0.1 mM	0.099±0.005 min^{-1}
L-U4	5U	t2M	0.1 mM	0.178±0.019 min^{-1}
L-U4	5U	t4M	0.1 mM	0.418±0.031 min^{-1}
L-U4	5U	$t4M^{Me}$	0.1 mM	0.080±0.010 min^{-1}
L-GU3	wt	None	0.1 mM	0.049±0.005 min^{-1}
L-GU3	UU	None	0.1 mM	0.323±0.036 min^{-1}
wt (L-GUGA)	wt	None	0.1 mM	0.603±0.026 min^{-1}

S5. Compound Characterization.
NMR spectra.

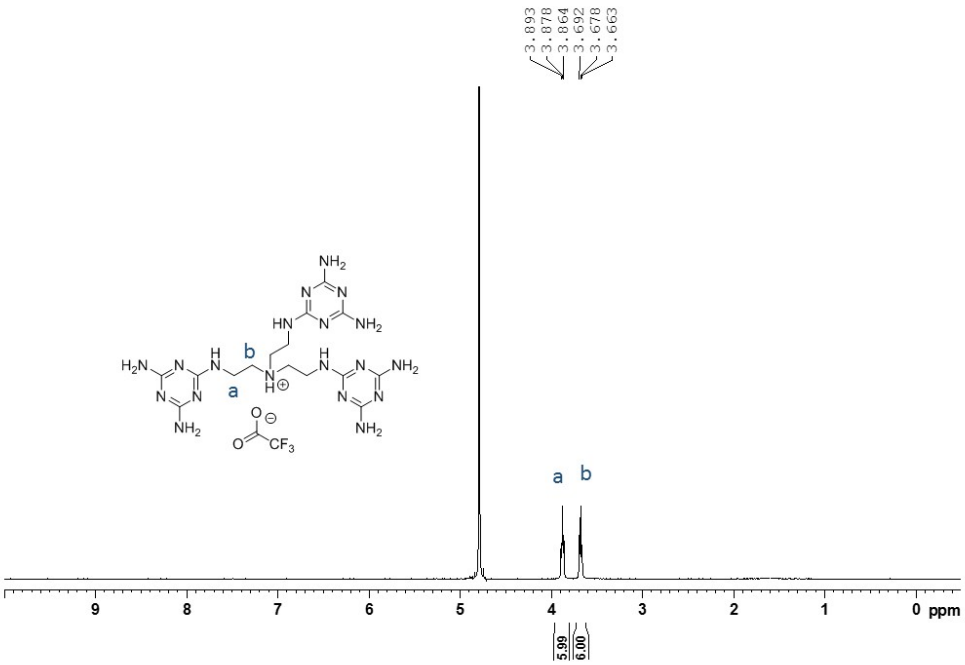


Figure S5.1 ^{1}H NMR (400 MHz, D_2O, pH=3) of t3M (**1**). TFA from HPLC.

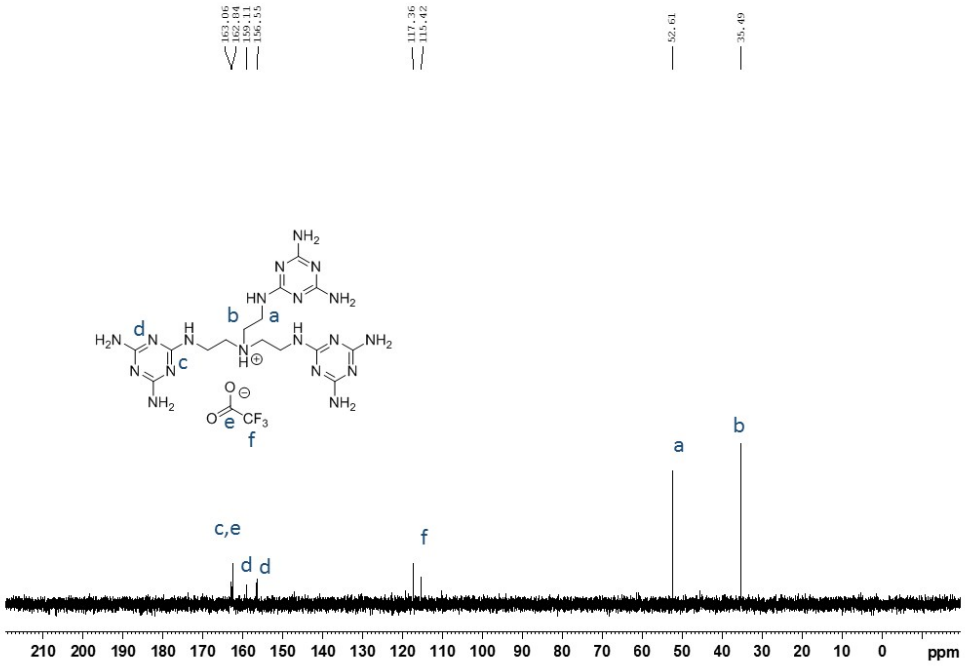


Figure S5.2 ^{13}C NMR (100 MHz, D_2O) of t3M (**1**). Two d peaks are possibly due to different environments of the protonated triazine rings at pH 3 (TFA from HPLC). The peaks coalesce after basification to pH 6.0 (Figure S5.4). Similar phenomenon was seen in t2M and t4M NMR spectra, with the expected spectral signatures observed at pH 6.0 (below).

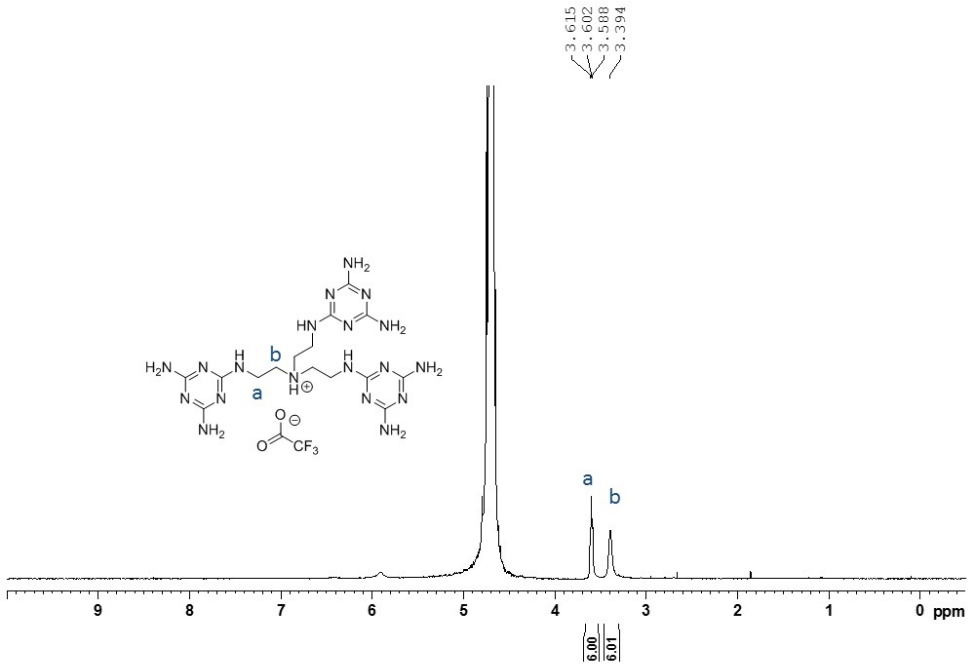


Figure S5.3 ^{1}H NMR (400 MHz, D_2O+NaOD, pH=6) of t3M (**1**). TFA from HPLC.

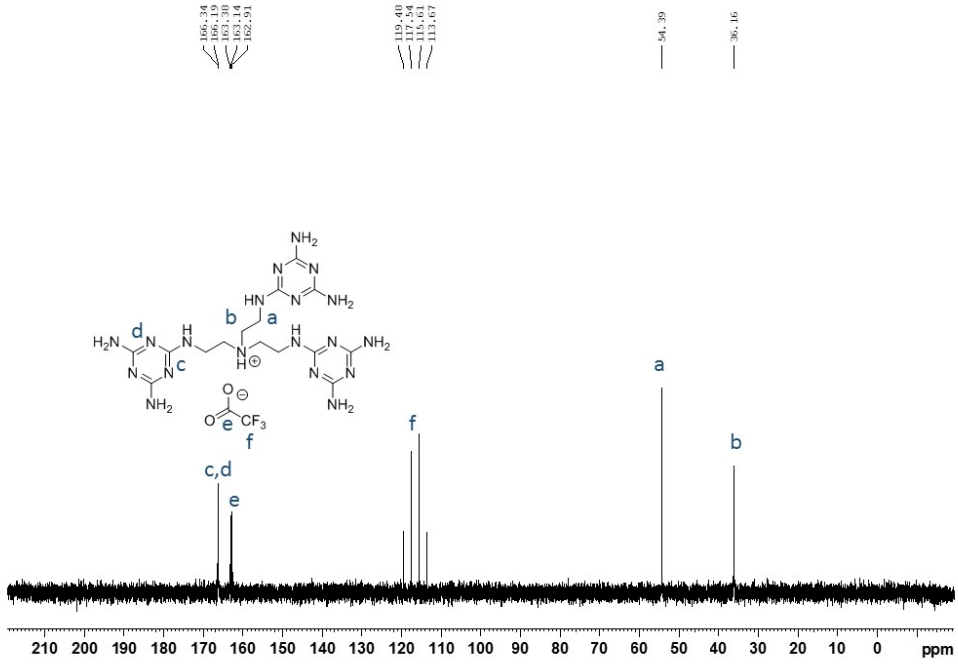


Figure S5.4 ^{13}C NMR (125 MHz, D_2O+NaOD, pH=6) of t3M (**1**). TFA from HPLC.

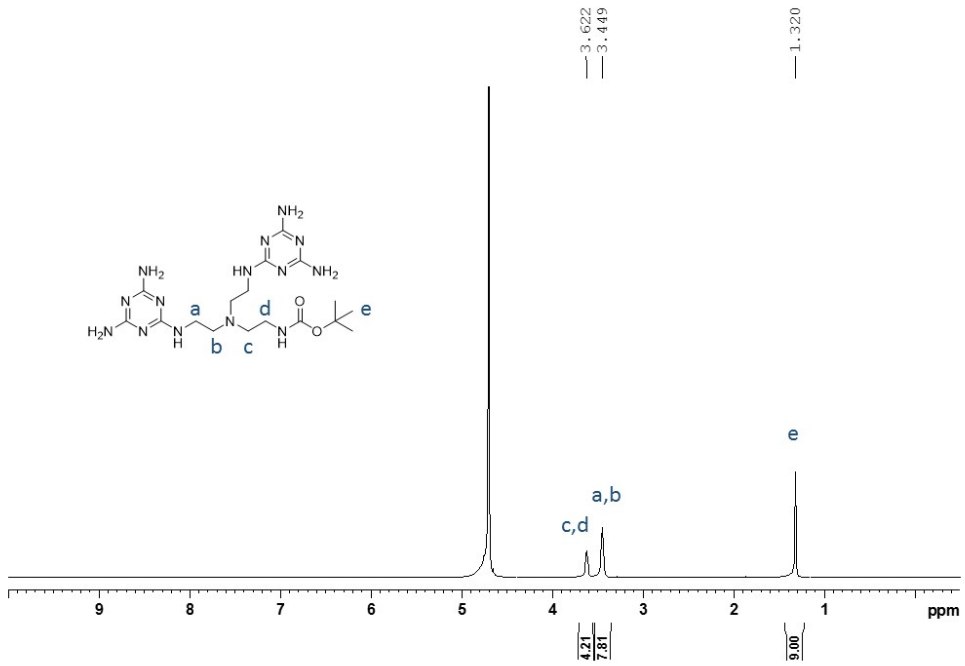


Figure S5.5 ^{1}H NMR (400 MHz, D_2O) of Boc t2M.

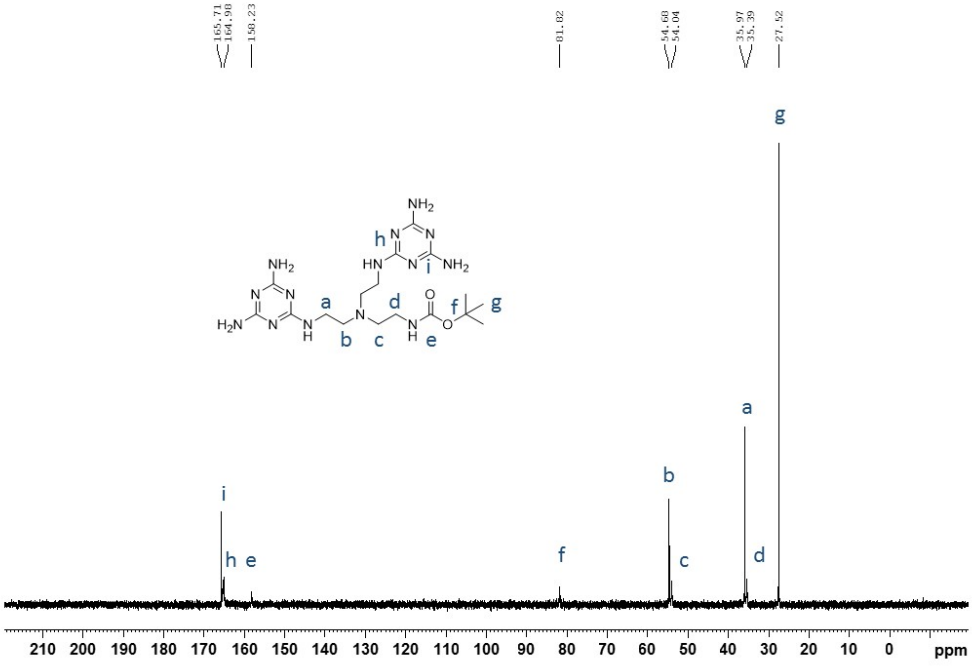


Figure S5.6 ^{13}C NMR (100 MHz, D_2O) of Boc t2M.

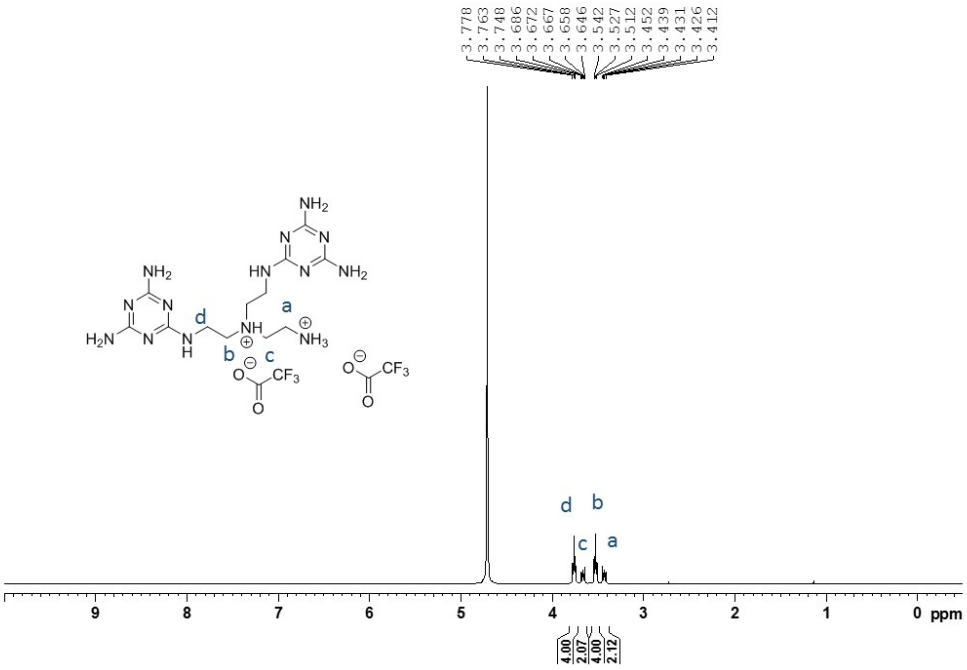


Figure S5.7 ^{1}H NMR (400 MHz, D_2O, pH=3) of t2M (**2a**). TFA from HPLC.

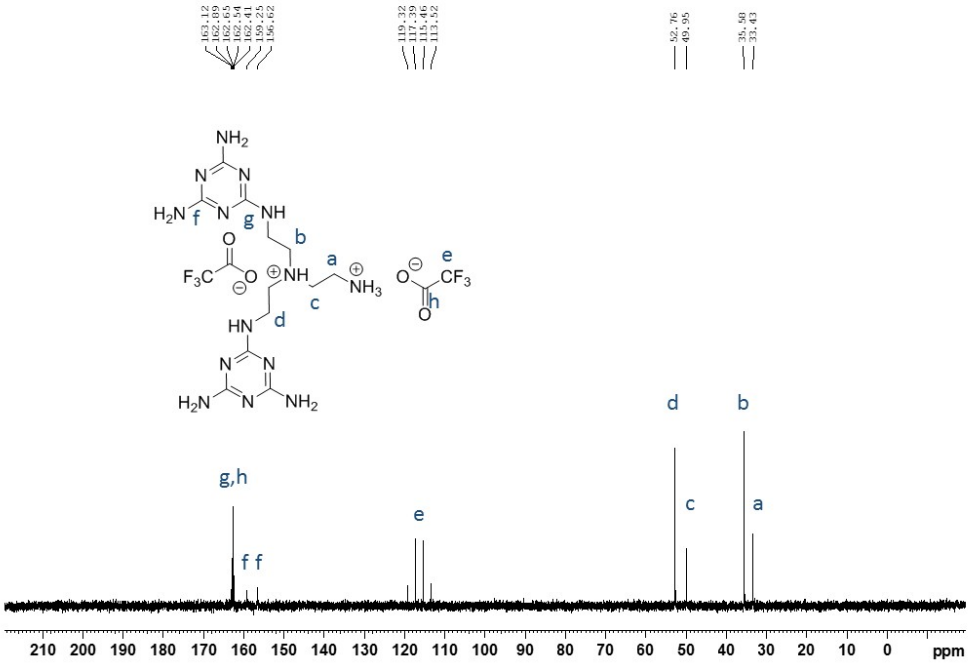


Figure S5.8 ^{13}C NMR (125 MHz, D_2O, pH=3) of t2M (**2a**). TFA from HPLC.

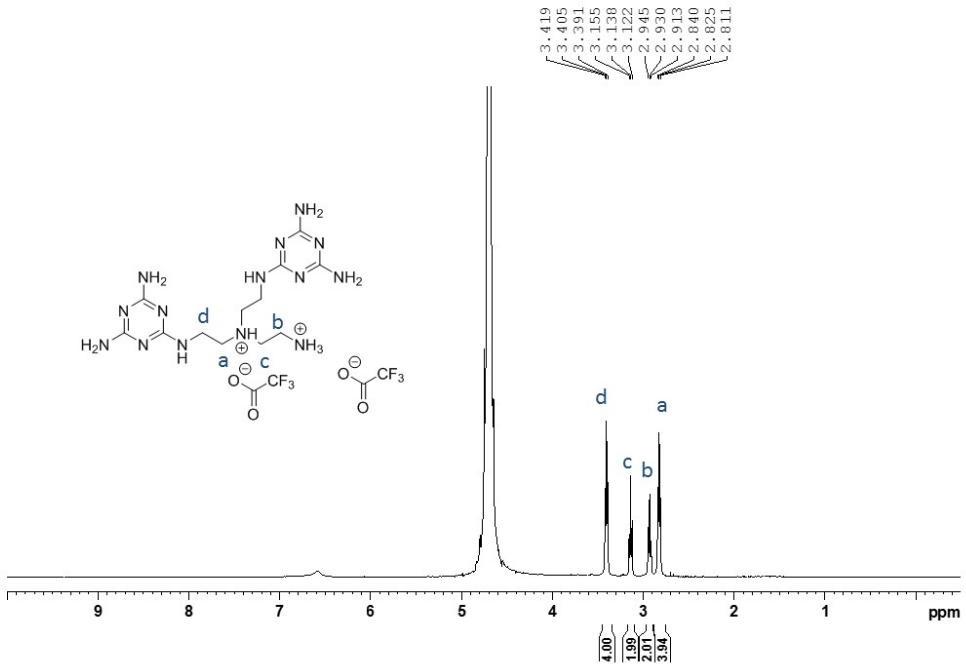


Figure S5.9 ^{1}H NMR (400 MHz, D_2O+NaOD, pH=6) of t2M (**2a**). TFA from HPLC.

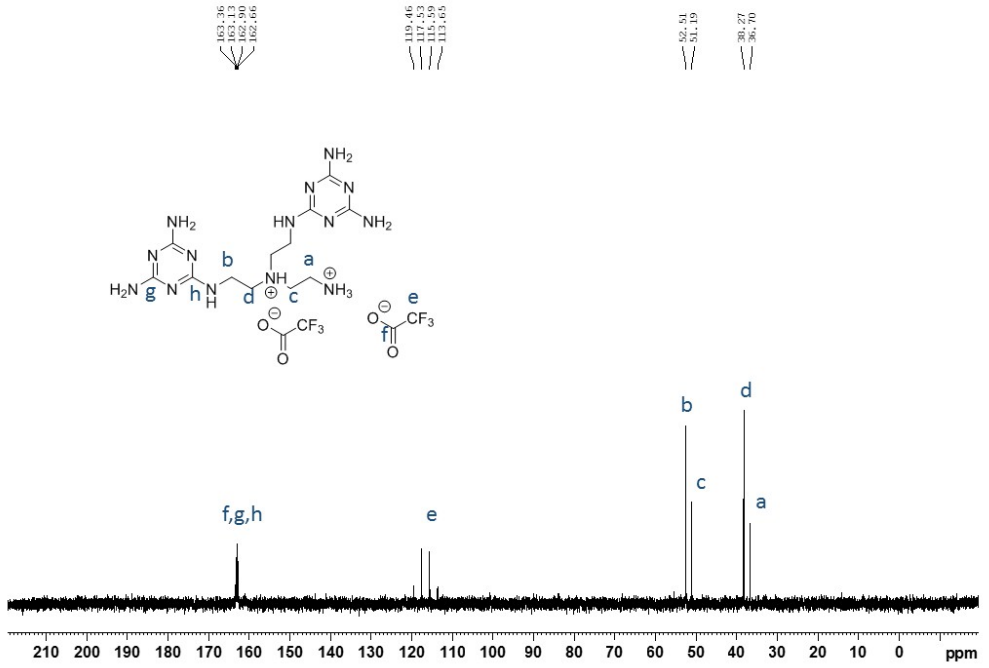


Figure S5.10 ^{13}C NMR (125 MHz, D_2O+NaOD, pH=6) of t2M (**2a**). TFA from HPLC.

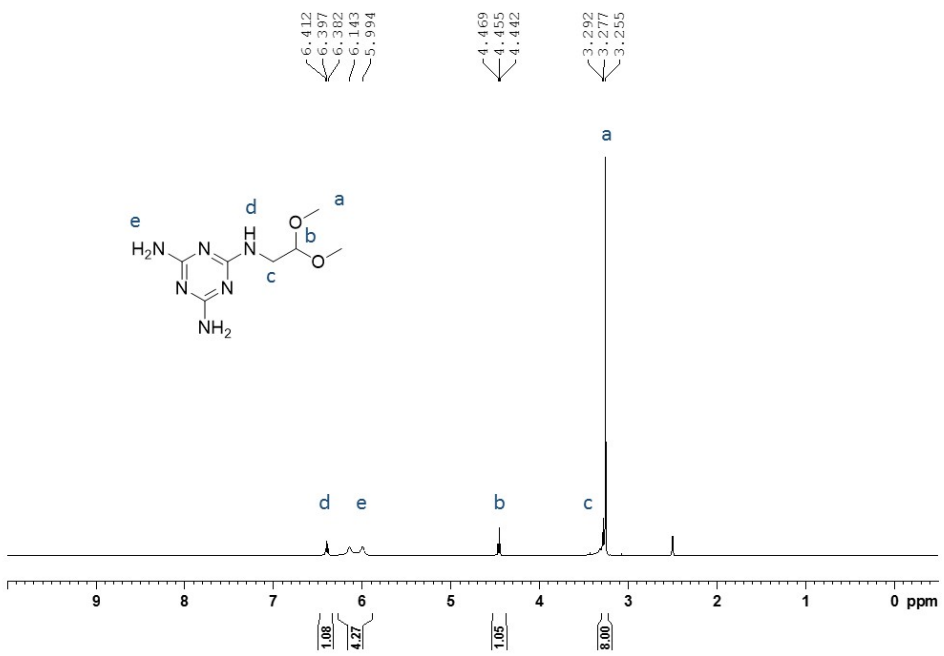


Figure S5.11 ^{1}H NMR (400 MHz, d_6-DMSO) of **4**.

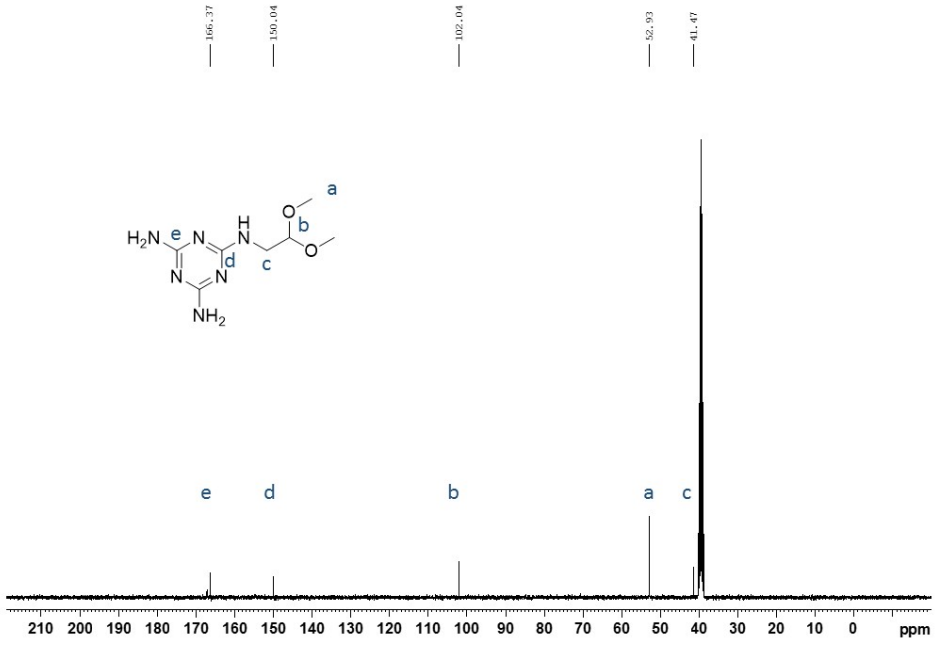


Figure S5.12 ^{13}C NMR (100 MHz, d_6-DMSO) of **4**.

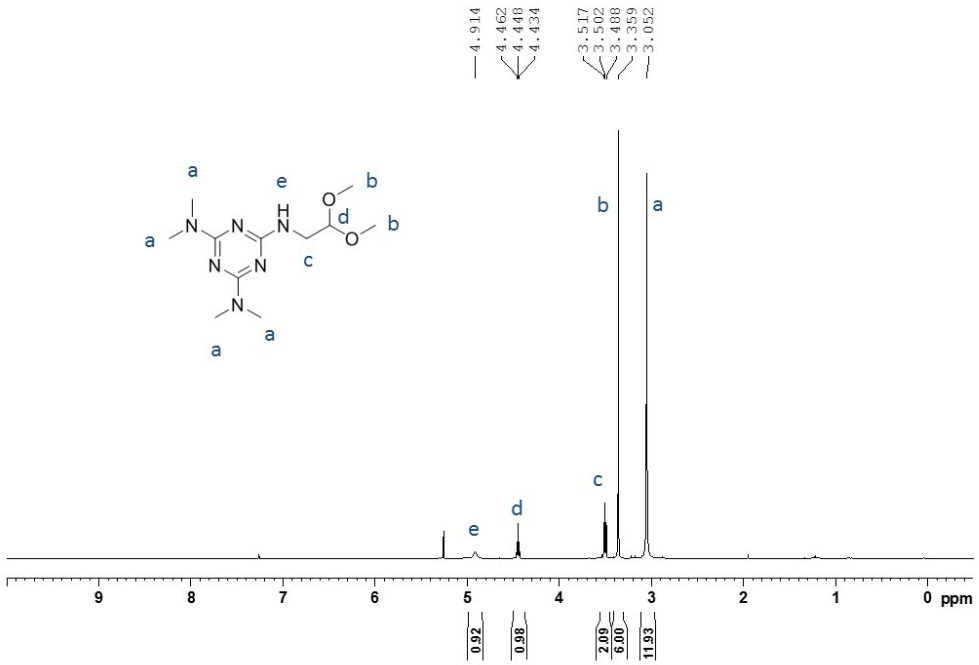


Figure S5.13 ^{1}H NMR (400 MHz, $CDCl_3$) of **5**.

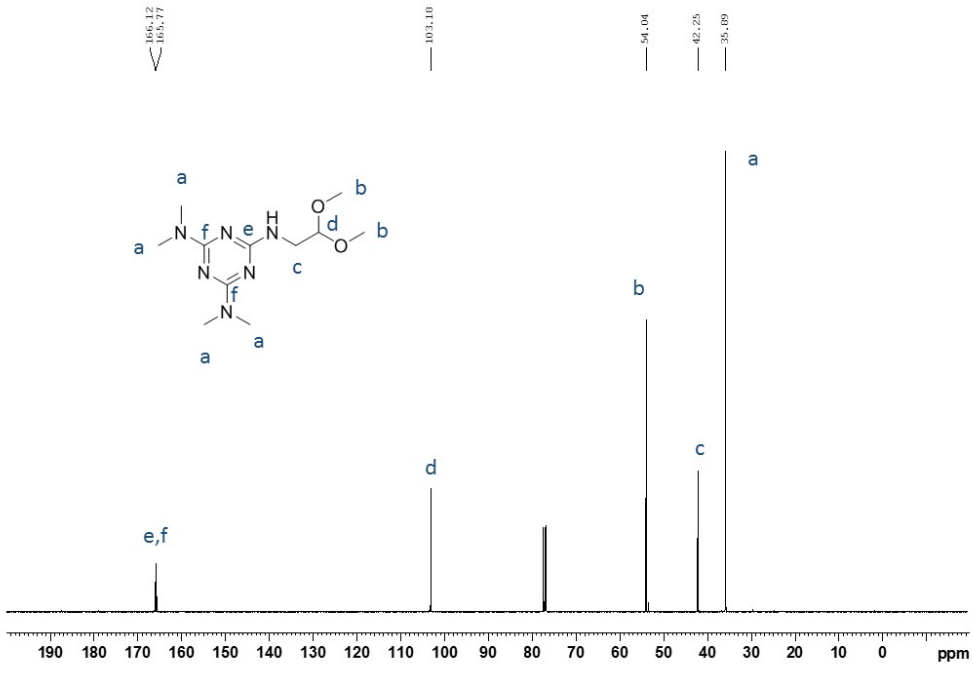


Figure S5.14 ^{13}C NMR (100 MHz, $CDCl_3$) of **5**.

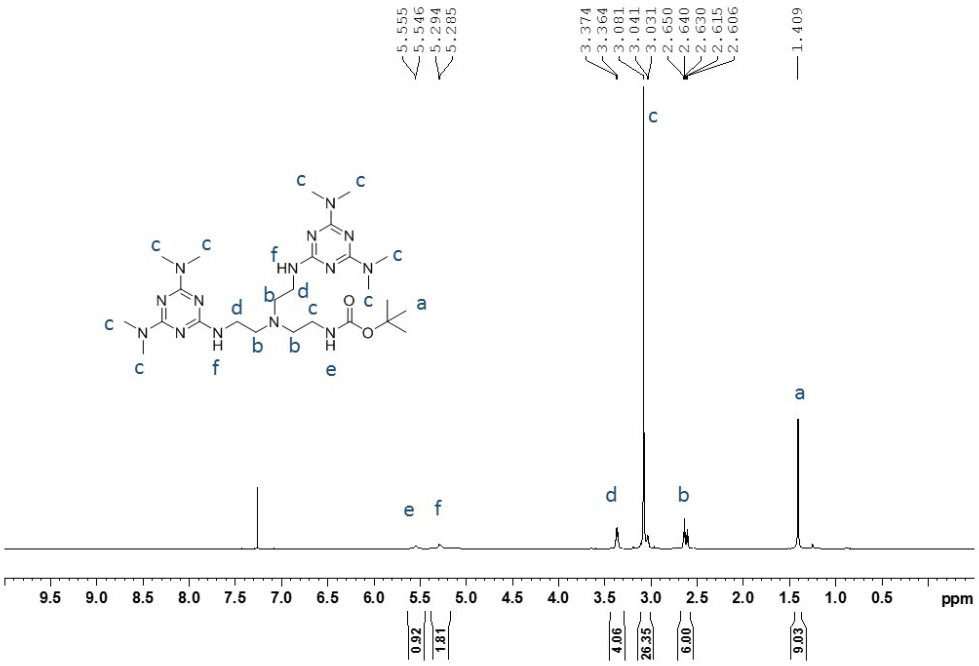


Figure S5.15 ^{1}H NMR (400 MHz, $CDCl_3$) of Boc t2M^{Me}.

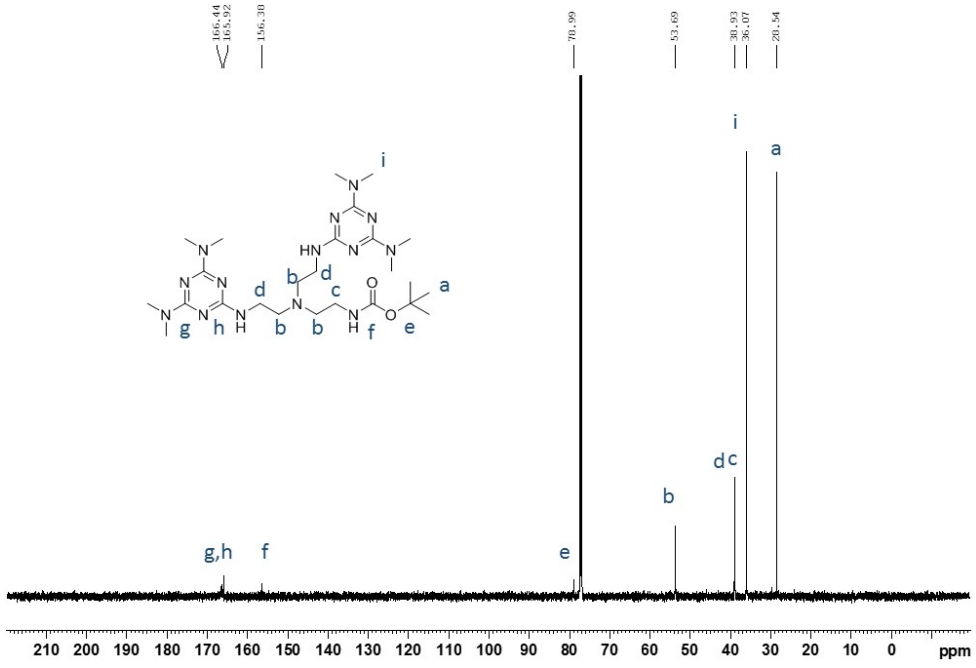


Figure S5.16 ^{13}C NMR (100 MHz, $CDCl_3$) of Boc t2M^{Me}.

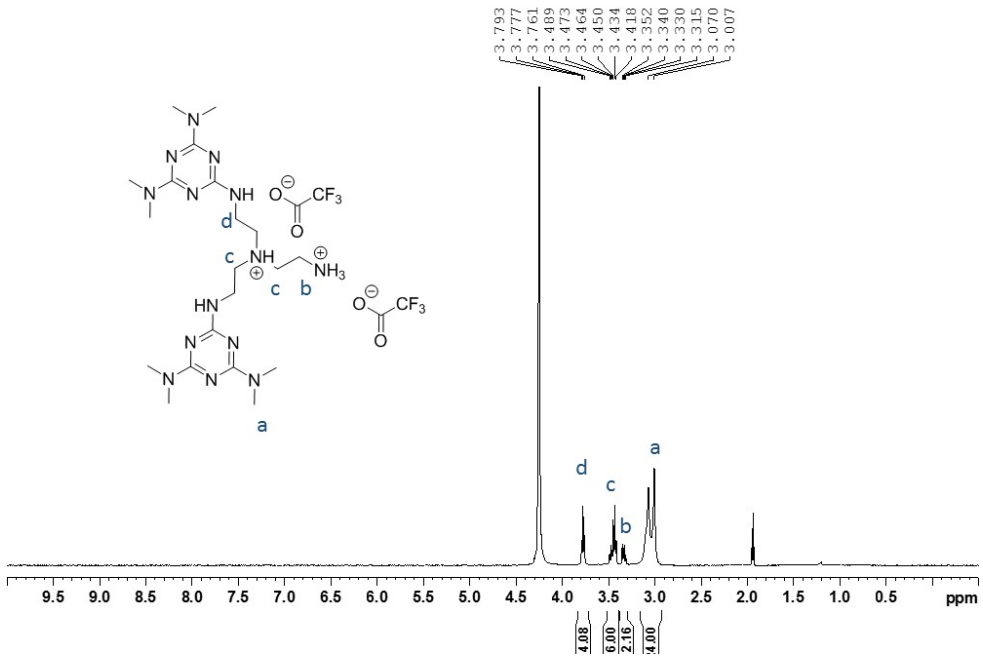


Figure S5.17 ^{1}H NMR(400 MHz, CD_3CN:D_2O=1:1) of t2M^{Me} (**2b**). TFA from deprotection of Boc.

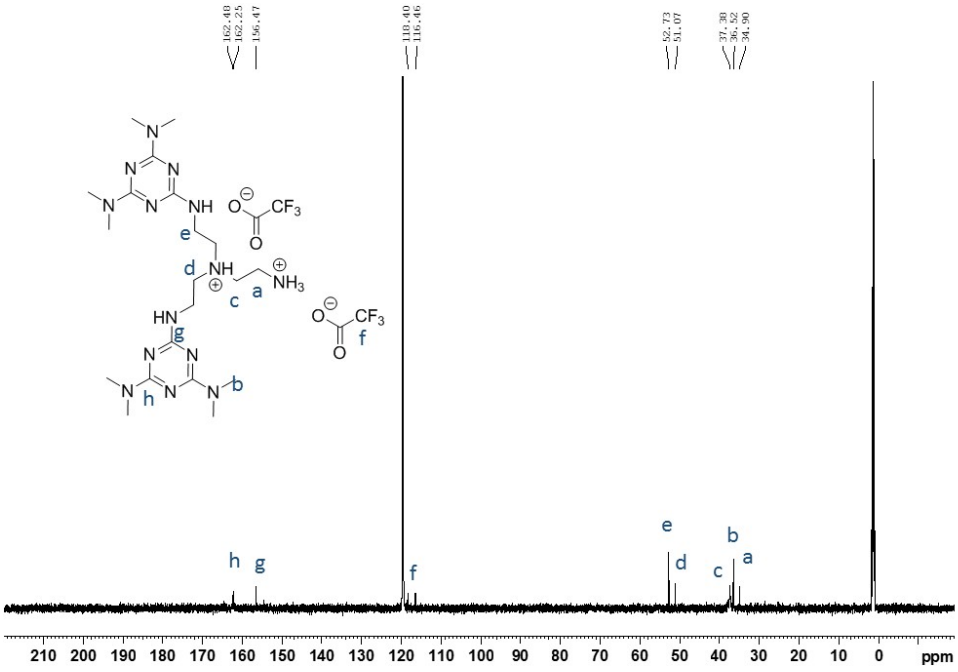


Figure S5.18 ^{13}C NMR(125 MHz, CD_3CN:D_2O=1:1) of t2M^{Me} (**2b**). TFA from deprotection of Boc.

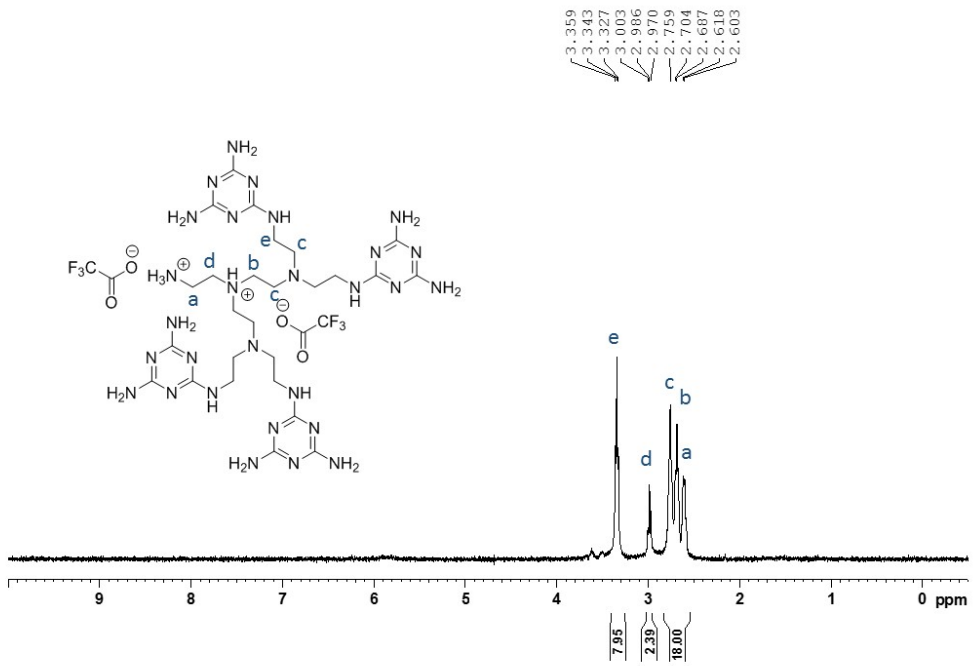


Figure S5.19 ^{1}H NMR (400 MHz, D_2O, water suppression, pH=6) of t4M (**6**). TFA from HPLC. Peak assignments by analogy to t3M and t2M.

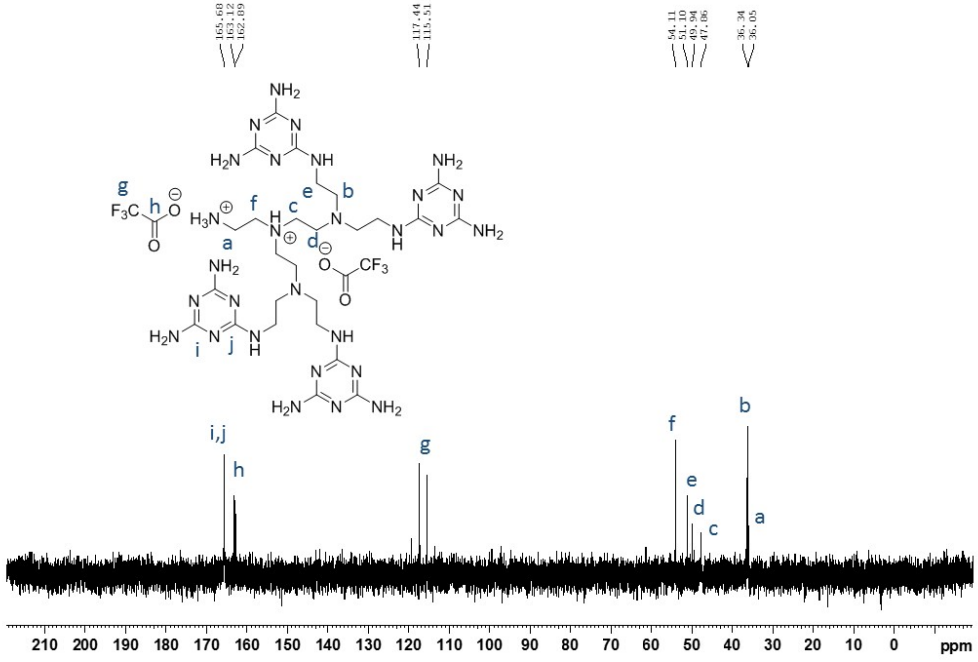


Figure S5.20 ^{13}C NMR (125 MHz, D_2O+NaOD, pH=6) of t4M (**6**). TFA from HPLC. Peak assignments by analogy to t3M and t2M.

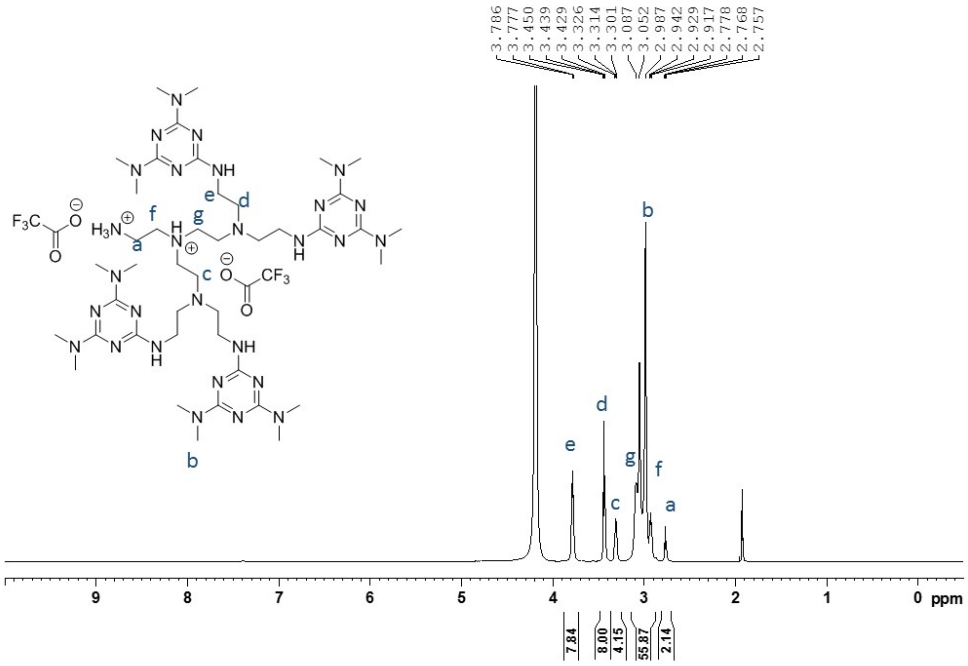


Figure S5.21 ^{1}H NMR (600 MHz, CD_3CN:D_2O=1:1) of t4M^{Me} (**7**). TFA from deprotection of Boc. Peak assignments by analogy to t3M and t2M.

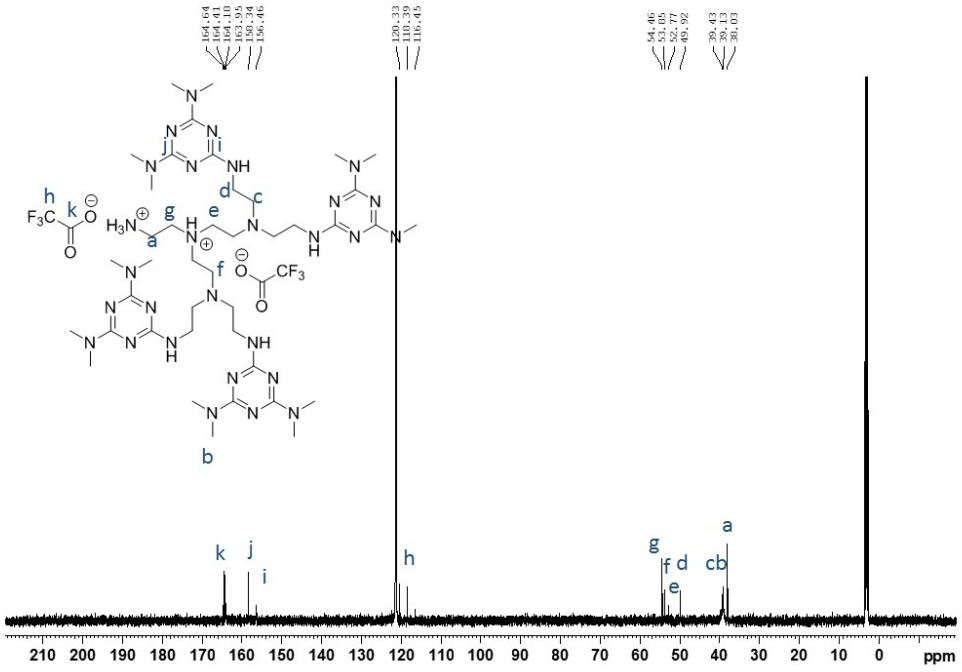


Figure S5.22 ^{13}C NMR (125 MHz, CD_3CN:D_2O=1:1) of t4M^{Me}(**7**). TFA from deprotection of Boc. Peak assignments by analogy to t3M and t2M.

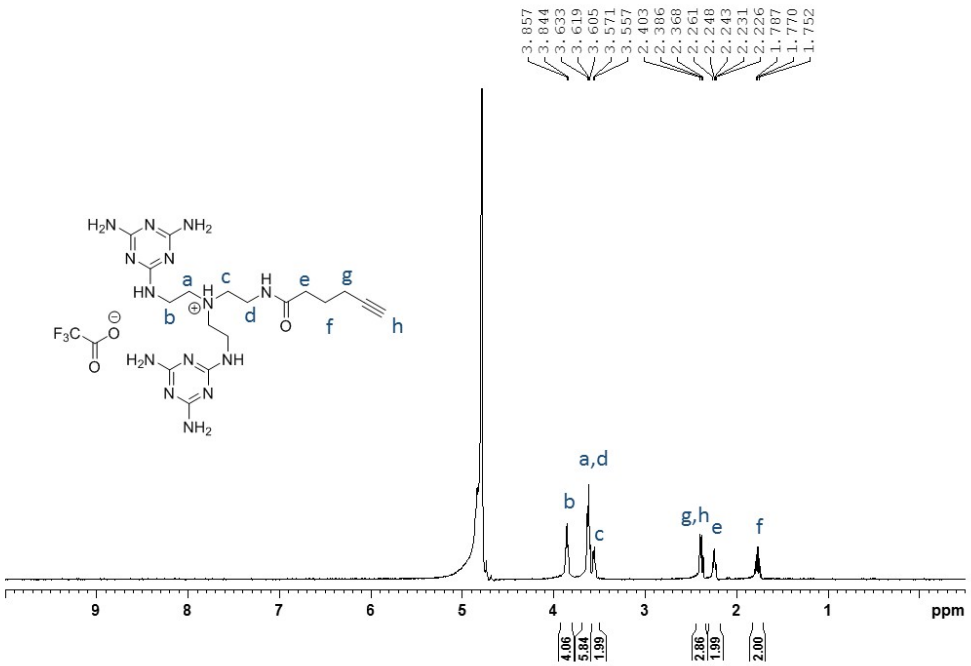


Figure S5.23 ^{1}H NMR (400 MHz, D_2O, pH=3) of t2M alkyne (**3**). TFA from HPLC.

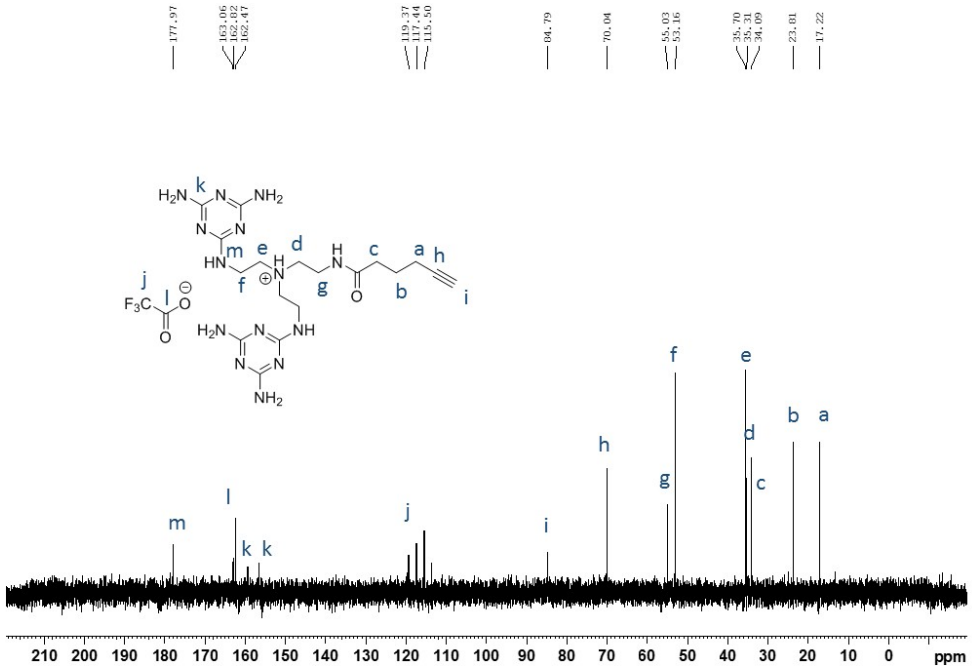


Figure S5.24 ^{13}C NMR (125 MHz, D_2O, pH=3) of t2M alkyne (**3**). TFA from HPLC.

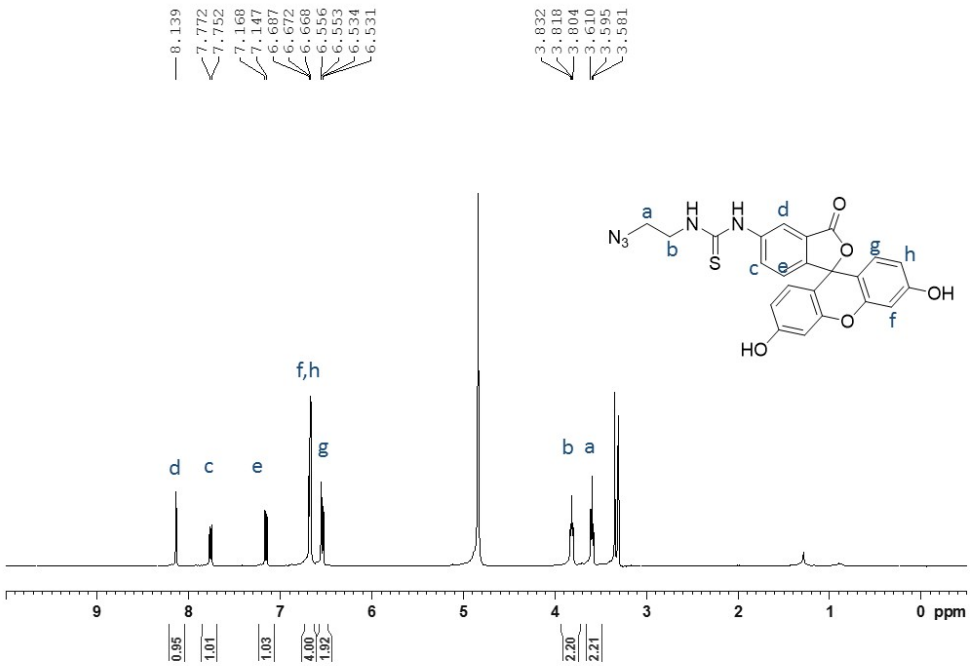


Figure S5.25 ^{1}H NMR (400 MHz, d_4-MeOD) of FITC N_3.

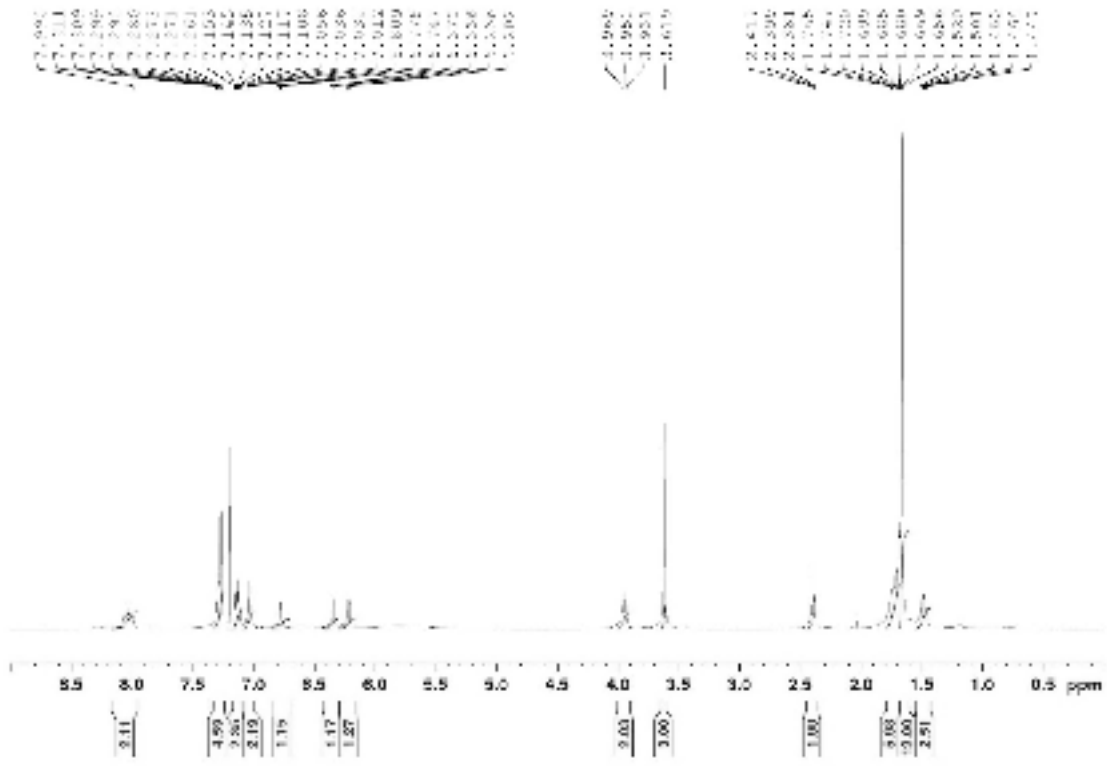


Figure S5.26 ^{1}H NMR (400 MHz, $CDCl_3$) of Cy5 Free acid.

HPLC and ESI Spectra.

HPLC traces are obtained on an reverse phase C18 analytical column with solvent A= 99% H_2O, 1% MeCN and 0.1% TFA and solvent B= 10% H_2O, 90% MeCN and 0.07% TFA. Flow rate of HPLC is 1 mL/min.

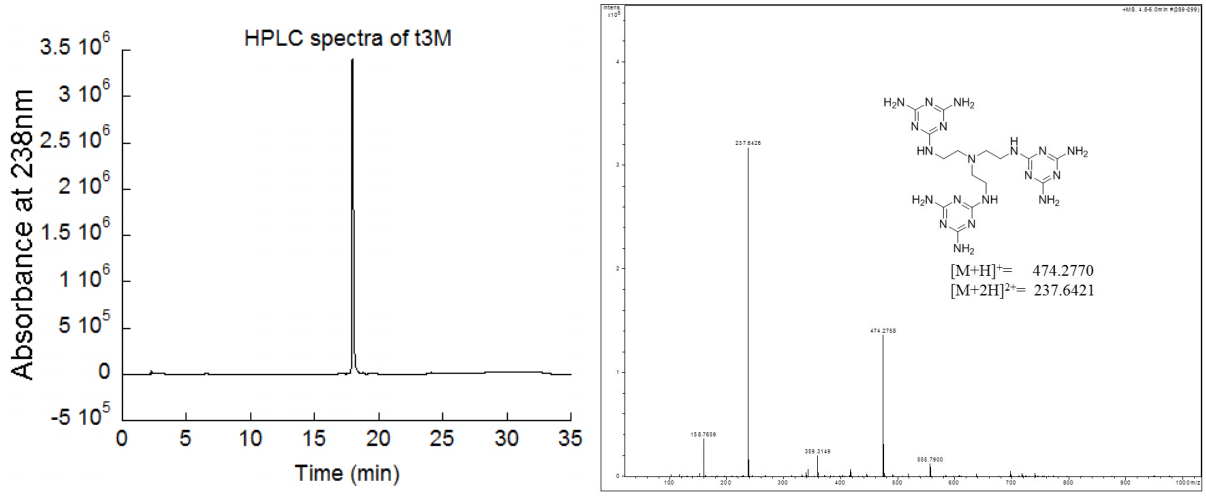


Figure S5.25 (Left) HPLC trace of t3M (**1**). Gradient: 0-5 min 0% solvent B, 5-15 min; linear gradient from 0 to 30% solvent B; 15-20 min, 30% solvent B; 20-22 min, linear gradient from 30 to 100% solvent B; 22-27 min, 100% solvent B; 27-29 min, linear gradient from 100 to 0% solvent B; 29-35 min, 0% solvent B. (Right) ESI spectra of t3M, peaks observed:474.2788, 237.6426.

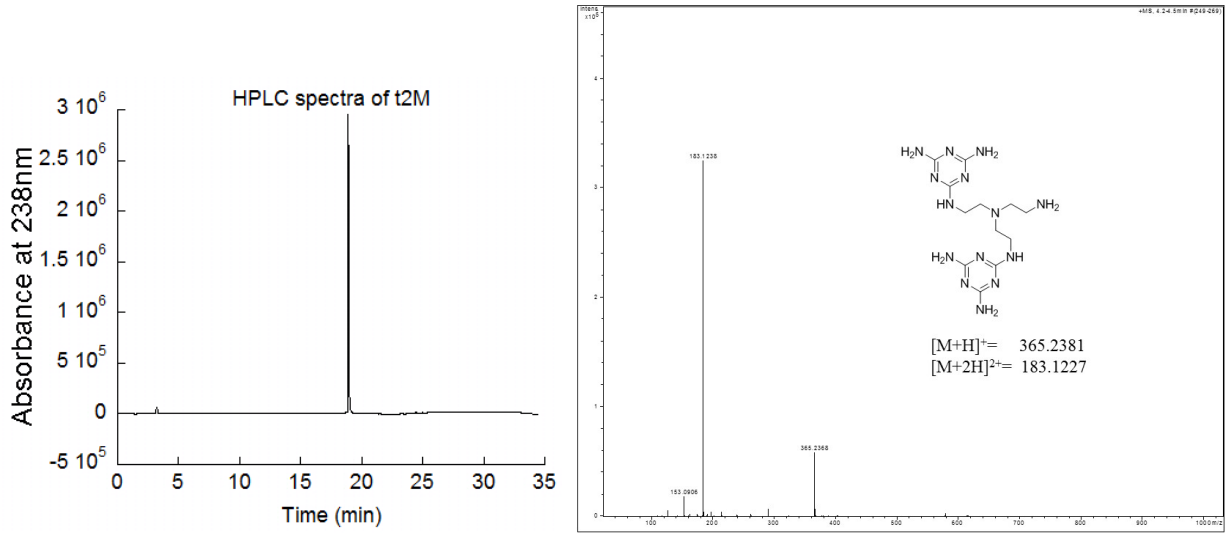


Figure S5.26 (Left) HPLC trace of t2M (**2a**). Gradient: 0-5 min 0% solvent B, 5-15 min; linear gradient from 0 to 30% solvent B; 15-20 min, 30% solvent B; 20-22 min, linear gradient from 30 to 100% solvent B; 22-27 min, 100% solvent B; 27-29 min, linear gradient from 100 to 0% solvent B; 29-35 min, 0% solvent B. (Right) ESI spectra of t2M, peaks observed:365.2368, 183.1238.

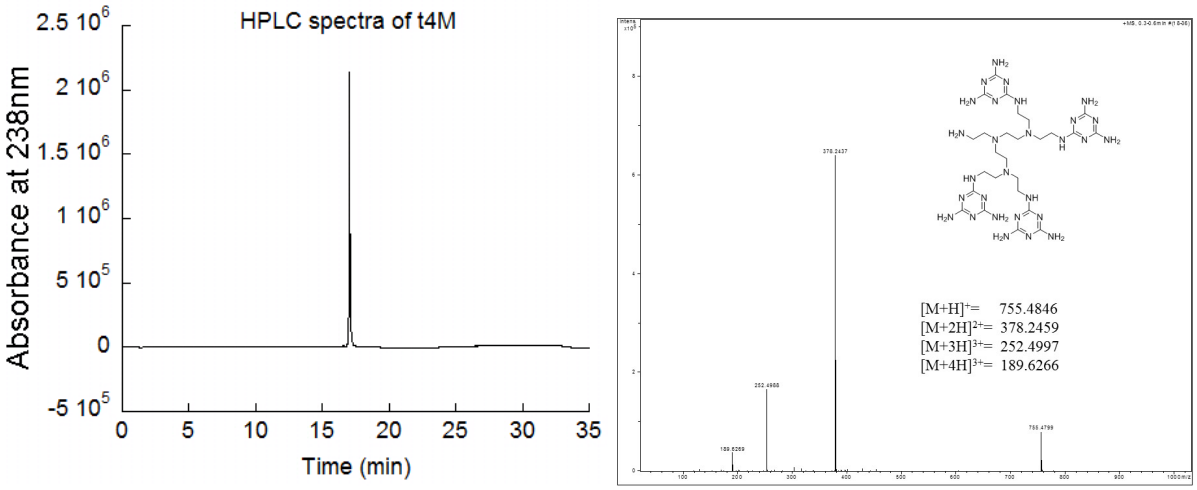


Figure S5.27 (Left) HPLC trace of t4M (**6**). Gradient: 0-5 min 0% solvent B, 5-15 min; linear gradient from 0 to 30% solvent B; 15-20 min, 30% solvent B; 20-22 min, linear gradient from 30 to 100% solvent B; 22-27 min, 100% solvent B; 27-29 min, linear gradient from 100 to 0% solvent B; 29-35 min, 0% solvent B. (Right)ESI spectra of t4M, peaks observed:755.4799, 378.2437, 252.4988, 189.6269.

S6. References:

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